

APPLYING SKELETAL, HISTOLOGICAL AND MOLECULAR TECHNIQUES

**APPLYING SKELETAL, HISTOLOGICAL AND MOLECULAR TECHNIQUES
TO SYPHILITIC SKELETAL REMAINS FROM THE PAST**

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ABSTRACT

Many have been searching for and contemplating the origins of syphilis. By understanding its emergence as a human pathogen we will be better able to elucidate its evolution through time and space as well as shed light on its current state. Ancient DNA techniques used to isolate *Treponema pallidum* subspecies *pallidum* DNA from archaeological human specimens provides direct evidence of its existence in the past. To date, only Kolman et al. (1999) have been successful in this endeavour. Along with this protocol, two other published protocols and novel allele specific techniques this thesis aims to add new cases of venereal syphilis identification from historic human remains. To accomplish this, sixteen skeletal samples from different time periods and geographic locations were collected for this project. Of importance are those dating from the Civil War time period from the United States as medical documents state these individuals suffered and/or died from the complications of syphilis. Samples from the United Kingdom are also critical to this analysis as they have confirmed pre-Columbian dates.

Along with attempts to isolate bacterial DNA, endogenous DNA (mitochondrial and amelogenin) is analyzed to provide an idea of the different levels of molecular preservation. General preservation as well as the identification of syphilis are also performed using microscopic techniques. By using a tiered approach (macroscopic to microscopic to molecular), a better idea of both preservation and disease presence can be ascertained. Results indicate that although syphilis could be identified at both the macroscopic and microscopic levels for some individuals and endogenous DNA was present, treponemal DNA failed to amplify. Many different reasons are suggested, for example poor conservation methods, misdiagnosis and diagenesis, but the most important possibility is the lack of bacterial DNA in bone at later stages of syphilis which was confirmed using the rabbit model. As a result, the present techniques may not be conducive for treponemal DNA isolation from ancient human remains.

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CHAPTER ONE:

INTRODUCTION

The origins of venereal syphilis have been passionately debated for centuries, but three hypotheses surrounding the emergence of the disease have dominated the debate: the Columbian, pre-Columbian and the Unitarian explanations. Generally, these hypotheses suggest that syphilis originated either in the New World, the Old World or was present in both regions but evolved to accommodate different geographical and sociological environments, respectively. Each of these hypotheses has its own merits, but only one should be most consistent with the available data. It is believed that a unified hypothesis might be developed one day if archaeological skeletal sources displaying putative syphilis (or other treponemal diseases e.g., yaws, bejel) could be tested by extracting, amplifying and comparing DNA sequences with modern clinical isolates of the *Treponema* bacteria.

Syphilis belongs to the family of spirochetes called the *Spirochaetaceae* and the genus *Treponema*, which includes four human pathogens and at least six human nonpathogens (Norris and Larsen, 1995). The pathogenic species are *T. pallidum* subsp. *pallidum* (venereal syphilis), *T. pallidum* subsp. *endemicum* (endemic syphilis or bejel), *T. pallidum* subsp. *pertenue* (yaws) and *T. carateum* (pinta). At present, the genetic information implies they are separate species with a common ancestor. For ease for both the reader and the writer, when “syphilis” is used in the remainder of this text, only venereal syphilis and not endemic syphilis is being discussed. Of the four human pathogens, only the first three are important to paleopathologists as they can alter bone in

relatively characteristic fashions. However syphilis has gained the name the ‘Great Imitator’, as both its clinical pathogenesis and skeletal alterations are quite similar to other diseases. For example, clinically syphilis can be confused with other sexually transmitted infections (e.g., gonorrhea) and skeletally it can be misdiagnosed as tuberculosis, leprosy, hematogenous osteomyelitis and Paget’s disease. As a result, researchers need to depend on a number of tactics to help in the differential diagnosis.

Samples used in this project originate from different areas of the world and from different time periods, encompassing both pre-Columbian and historic eras. Nine samples come from individuals who lived during the Civil War time period (1861-65) and are from a collection housed by 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 living in the mid 1800s.

The samples dating from around the Civil War period are important to this research 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 medical practitioners living during this time period. The remaining skeletal samples only provide a putative presence of syphilis via skeletal alterations.

One would think that testing known samples first with an ancient DNA (aDNA) approach would reveal if the protocol is working properly (i.e., it is identifying syphilis).

Then if treponemal DNA could be amplified from both pre-Columbian and post-Columbian materials, it would allow for a comparative analysis of syphilis' presence in both the Old and New Worlds. It might also allow for the analysis of the evolution of the disease from a non-sexually transmitted disease to a sexually transmitted disease.

The first issue concerning our understanding of syphilis and the feasibility of this study pertains to whether the *Treponema* bacteria will disseminate to the skeletal matrix. Although the presence of preserved treponemal organisms is of a contentious nature, spirochetes have been found in bone 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. He goes on to say 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 degenerate. 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 jaw bones (e.g., tooth germ, pulp, uncalcified and calcified dentine, dental follicle and enamel epithelium) of a macerated fetus with congenital syphilis. It is quite possible that the researcher was observing *T. 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 my knowledge, no new studies have been able to duplicate this 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.

Today, researchers are still interested in providing proof of the presence of *T. pallidum* during different stages of infection, but they are approaching it from another angle: DNA. Use of the Polymerase Chain Reaction (PCR) in clinical research has the advantage of being able to target *T.p. subsp. pallidum* DNA specifically, even in the later stages of infection. For example, Marfin et al. (2001) were able to detect treponemal DNA (using the Polymerase I gene) in eight out of 13 samples of latent syphilis*. Their study helps to demonstrate that “spirochetemia can occur throughout the course of *T. pallidum* infection” (Marfin et al., 2001:163). Pietravallo et al. (1999) were also successful in isolating treponemal DNA (from sera) from individuals with latent syphilis and even from those who had received antimicrobial drugs in the past two weeks, but not from those with approximately three years lag from penicillin therapy. In another study performed by Hollier et al. (2001), PCR was able to detect DNA from amniotic fluid taken from mothers in different stages of the disease, including the early latent stage (4/5 were successful). Since *T. pallidum* DNA can be isolated during later stages of disease, it stands to reason that it should be possible to isolate it from skeletonized individuals displaying bone lesions.

The hypothesis that detection of treponemal DNA is possible in archaeological remains also stems from earlier studies using paraffin-embedded tissue. Testicular tissue

* Although Marfin et al. (2001:165) do admit the possibility exists that these individuals could have been in the secondary stage due to no standardized definition for those persons with indistinct signs or symptoms of secondary disease.

from syphilitic rabbits was fixed in 10% phosphate-buffered Formalin and later (no time line given by authors) successfully isolated *T. pallidum* DNA (Burstain et al., 1991). These authors go on to predict that archival specimens could be used to help “resolve a number of questions concerning syphilis in the preantibiotic and perhaps even the prehistoric eras” (*Ibid.*: 66). Zöchling et al. (1997) also present an impressive study using formalin-fixed, paraffin-embedded biopsy specimens from skin lesions of patients with secondary and tertiary syphilis. 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, Zöchling et al. (1997) amplified a very small fragment (196 bp) via a nested PCR (original target was of a 379 bp fragment from the 47-kDa membrane protein) technique which resembles aDNA methodologies.

These successful PCR amplifications from patients and archival samples representing early to latent stages of syphilis provide good ammunition for the possible success one could achieve in isolating it in human skeletal remains with lesions indicative of later stage syphilis infection. However it should be pointed out that the lack of 100% success rates even from modern clinical samples, suggests that isolating *T. pallidum* DNA from archaeological samples will be extremely difficult. This is based on the fact that *T. pallidum* bacteria are extremely fragile organisms and do not survive long outside of their

‘living’ human host (Cates, 1998; Florde, 1994; Pusey, 1933; Radolf et al., 1989; Schouls, 1992; van der Sluis et al., 1984).

Attempts to isolate treponemal (syphilitic) DNA from ancient human material have been undertaken. It was first tried on muscle tissue from mummified individuals expressing tertiary treponematoses excavated from El Morro, Arica and San Miguel de Azapa, Chile (Rogan and Lentz, 1995). Rogan and Lentz (1995) 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*). 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) 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 of an individual expressing saber shins from

[♦] However, it must be kept in mind that other skin diseases could have used similar treatments.

Easter Island (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 grams of femoral bone and RFLP (Restriction Fragment Length Polymorphism) analysis to distinguish the species. Although successful, the Kolman et al. (1999) approach is highly problematic in a number of ways. The most unsettling is the use of such large quantities of bone as most ancient DNA work uses less than one gram of material to extract DNA. As aDNA is an invasive, destructive technique it often means access to samples, especially of human origin, is restricted or denied. However, acceptance of tissue removal does occur when researchers assure museum curators that minimal amounts will be taken. In consequence, use of such a large quantity of bone by Kolman and colleagues makes their research nonreplicable.

Another problem with their approach is that a sensitivity level for the PCR reaction was not provided, although they specified that the PCR conditions were optimized using *T. p.* subsp. *pallidum* Nichols strain. Without knowing at what point the protocol ceases to detect treponemal DNA, researchers may search endlessly within their own samples if the amount of DNA present actually falls below this detection limit. Finally, although RFLP analysis is a confirmatory tool as only those sites recognized by the restriction enzyme are cut, it adds another step in the identification process.

The purpose of this thesis is to contribute to the field of paleopathology by undertaking a holistic examination of the presence of putative syphilis in human remains of antiquity. The lack of completely distinct macroscopic skeletal lesions which could set syphilis apart from other diseases (including yaws, endemic syphilis, tuberculosis and

leprosy, to name a few) means that paleopathologists can only make putative diagnoses of the disease. To help rectify this, histological techniques (after Schultz, 1994, 2001 and 2003) were used as another avenue to help confirm the diagnosis of syphilis in the samples studied for this project. Finally, aDNA methodologies were used to attempt to identify syphilis at the molecular level. This thesis explores the many reasons why it may not be possible to study syphilis from an aDNA perspective.

In essence, this thesis serves as a warning for paleopathologists who hope that the new and exciting world of ancient DNA can help them more accurately diagnose 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.

1.1 Goals of the Study

Since 1999, no other group, including Kolman et al. (1999) (N. Tuross, personal communication), has been able to duplicate the Kolman et al. results, nor provide another molecular method to identify syphilis in ancient human remains. This thesis set out to take advantage of this opportunity.

The main objectives of this thesis project include:

1. Using and evaluating a comprehensive multi-component approach (i.e., a combination of skeletal, histological and molecular methodologies) for the identification of syphilis in human remains from the past.
2. Using and evaluating the paleohistomorphological criteria in the identification of syphilis set forth by Schultz (1994, 2001 and 2003).

3. Examining the preservation of bone micro-architecture and evaluating its correlation to molecular preservation.
4. Isolating human mitochondrial (mtDNA) and amelogenin DNA from the individuals sampled for this project to assess endogenous molecular preservation.
5. Employing Kolman et al.'s (1999) protocol, several other published protocols (Liu et al., 2001 and Zoechling et al., 1997) as well as novel allele specific methods to help in the analysis of treponemal DNA in human remains from the past.
6. Understanding why the aforementioned protocols used in this project to isolate the syphilis bacterium were unsuccessful, including looking at the organism in greater detail and using a rabbit model approach to evaluate the presence of treponemal bacteria in bone during different stages of the disease.

1.2 Synopsis by Chapter

This thesis consists of seven chapters. The second chapter presents the fundamentals of the three main theories concerning the origins of syphilis. This chapter then moves to the history of the disease from its early understandings of transmission, appearance and treatment, followed by a closer look at the pathogenesis of syphilis and a discussion of its tissue tropism. The second chapter ends by positioning the individuals sampled for this project into a sociocultural context to remind the reader of their status as once living people and the concerns about syphilis during the time period in which they lived.

The third chapter discusses the macroscopic skeletal manifestations and the microscopic characteristics of syphilis as well as potential candidates that could be part of the differential diagnosis for this disease. This chapter also presents a synopsis of the techniques used both in the clinical and research setting with a particular emphasis on molecular approaches.

The fourth chapter situates geographical and temporal backgrounds for the sampled individuals (N=16) used in this project. Detailed descriptions with some illustrations of the macroscopic and microscopic manifestations of syphilis are provided for each individual. The molecular section of this chapter explains how the samples were decontaminated and processed to isolate human mitochondrial, amelogenin (sex) and treponemal DNA. This section also describes the use of rabbit tissues representing different stages of induced syphilis as well as the production of clones required to understand the progression of the disease and the analysis of treponemal DNA, respectively. This chapter ends by setting out some of the limitations associated with this sort of project.

The fifth chapter starts with the histological results obtained from those samples that were amenable to this form of analysis (i.e., those with large enough bone samples to allow for both molecular and microscopic analyses; N=7). Histological bone analyses provided information concerning preservation of osteological micro-architecture, those samples affected by diagenesis (e.g., microorganism focal destruction) and the presence of structures (e.g., Polsters and Grenzstreifen) indicative of syphilis. Molecular results concerning human mitochondrial DNA and sexing are provided as well as the attempt at

isolating treponemal DNA from individuals sampled for this project. The chapter finishes with the results obtained from treponemal DNA isolated from rabbit material.

The sixth chapter presents a detailed discussion of the differential diagnoses potentially applicable to the pathological lesions identified on the individuals used in this project. The benefits of histological methods are described along with some criticisms. The correlation between the effects of diagenesis on bone structure and its constituents with molecular preservation is discussed. Organism fragility is then presented as a major potential stumbling block concerning the isolation of treponemal DNA in ancient human remains. Finally, the rabbit model experiment used in this project sheds light on the theory of bacterial invasion in bone.

The final chapter presents a summation of this project, a synopsis of the discussion as well as possible future research that would extend the information needed to carry on in the field of syphilis identification in ancient human remains. I implore people to gain as much information concerning their samples prior to aDNA analysis as conservation methods and burial environments may decrease the potential for well-preserved molecules; to use multiple approaches, even within a single methodology (e.g., look at multiple genes), when diagnosing disease in the past to provide a more accurate diagnosis; to investigate the preservation of other skeletal constituents (e.g., protein) prior to DNA amplification; to try amplifying other pathogens (e.g., *M. tuberculosis*) that cause similar skeletal alterations; and to increase sensitivity levels of the PCR reaction.

An appendix is also provided to the reader to illustrate the macro-micro histological thin bone sections prepared for this project.

CHAPTER TWO:

The HISTORICAL and MEDICAL ASPECTS of SYPHILIS and the SOCIAL ENVIRONMENT of the SAMPLED INDIVIDUALS USED IN THIS THESIS

The name “syphilis” can be attributed to a book written by Fracastoro in 1530 called *Syphilis sive morbus Gallicus* (Syphilis or the French disease) which described clinical features of the disease, recommended treatment and ended with a tale of a Shepard whom he called Syphilus (Oriol, 1994; Pusey, 1933). The tale tells of Syphilus offending the sun god Apollo who is then punished with a foul disease, but later exonerated and presented with a guaiacum tree and advice on the value of mercury. Syphilis as a form of punishment is a common theme in both the past and present experiences of this sexually transmitted disease.

It has been estimated that almost a twentieth of the population was infected with syphilis in Europe by 1495 (Oriol, 1994). The disease spread easily as armies accompanied by bands of prostitutes moved across the countryside during times of war and then transmitted the disease to their home country after they disbanded. From Europe, syphilis spread to India in 1498 and then to China in 1505 (Oriol, 1994; Parran, 1937) suggesting increased virulence due to its speed in distribution. By the end of the 16th century, syphilis specialists noted that the disease had become milder in presentation (Parran, 1937; Quéral, 1990) with less florid pustular eruptions and ulcerations (Sanchez and Luger, 1993). Although the number of cases of syphilis has decreased considerably since the introduction of penicillin, it is still an important sexually transmitted infection. For example on a world-wide basis, the World Health Organization estimated that 12 million new cases of syphilis occurred worldwide in 1999 with 90% of them found in

developing countries (Peeling and Mabey, 2004). In the U.S.A. in 1990 syphilis rates increased to 20 cases per 100,000 persons (Webster et al., 1991) from 3.9 cases per 100,000 in 1956 (Nakashima et al., 1996). The rate in Canada has remained low since the introduction of penicillin and in 1999 rates averaged around 0.6 per 100,000 (Health Canada: Population and Public Health Branch, 2002). It is more prevalent in urban locales, low socio-economic groups (although all socio-economic strata are at risk), certain occupations (transient workers e.g., miners [Kark, 2003], commercial sex workers, truck drivers), drug users and HIV positive patients (Cates, 1998; Sanchez and Luger, 1993; Thomas et al., 1999).

2.1 The Origins of Syphilis

Scholars from various backgrounds have debated the origins of syphilis with great passion. These debates hinge upon many pieces of evidence that range from historical reports and medical documents, archaeological remains and, now, genetic analyses of the spirochetes. By using these data three theories have emerged and have come to the forefront of discussions. These three theories, the Columbian, pre-Columbian and the Unitarian, attempt to explain the origins and spread of venereal syphilis throughout the world.

The Columbian hypothesis proposes that syphilis originated in the New World (Haiti) and was introduced to Spain by Columbus and his crew in 1493 (Crosby, 1969; Goff, 1967; Harrison, 1959; Pusey, 1915 and 1933). It is argued that the introduction of a new virulent disease to a susceptible population (i.e., one with no immunity to it) led to an epidemic of syphilis in Europe around AD 1495. The novelty of the disease created a

'name-blaming' scenario as it spread throughout Europe: the Neapolitan, Italian, French, German, Polish or Spanish Pox (Brothwell, 1970; Holcomb, 1937; Oriel, 1994; Parran, 1937; Pusey, 1915 and 1933; Quéral, 1990).

The Columbian hypothesis is supported by numerous examples of skeletal remains exhibiting lesions characteristic of treponemal disease in the New World (e.g., Baker and Armelagos, 1988; El-Najjar, 1979 and 2000; Gerszten et al., 1998; Pineda et al., 1998; Powell, 1991). According to those who propose this hypothesis, no skeletal remains should exist in Europe with lesions indicative of treponemal infection prior to 1492. This held true until relatively recently as excavations and a more concerted effort and interest in identifying syphilis have unearthed examples from the archaeological record. Human skeletal remains diagnosed with syphilitic lesions now exist from the Old World which have been firmly dated prior to Columbus' voyage in 1492 ([Scotland] Cardy et al., 1997; [Italy] Henneberg and Henneberg, 1994; [Ipswich and Rivenhall, England] Mays et al., 2003; [France] Pálfi et al., 1992; [Gloucester, England] Roberts, 1994; [Norwich, England] Stirland, 1991). Therefore, finding physical evidence of the presence of syphilis in Europe prior to 1492 indicates that the Columbian hypothesis can no longer be sustained. However, the paucity of cases may lead one to question why so little evidence has been found in the Old World archaeological record.

First of all it must be recognized that the burial population is only a fraction of the actual existing population with many factors affecting the preservation of skeletal remains. We may be seeing fewer examples of syphilis in past populations because their skeletal remains may not have survived the burial environment or some individuals may

not have been buried within regular ‘cemetery’ establishments that have been recognized as such. It is also possible that the higher virulence of the disease in the past resulted in people dying prior to bone manifestations. Steinbock (1976) and Hackett (1975) state that only a small percentage (<20%) of individuals will actually manifest skeletal lesions, thus implying that in the archaeological record the starting population with possible skeletal alterations indicative of syphilis will be rather small and when this is combined with preservation issues, the number will decrease even further. Throughout history people have also been exposed to and have died from many other ailments (e.g., other diseases [cholera, typhoid], accidental death, etc) which were acute in nature, adding to the possibility that syphilitic osseous lesions did not have the time to manifest prior to death. The combination of short life spans and long incubation periods for tertiary signs to manifest may have also been a factor. Finally and most importantly, there are still geographical areas that have not been excavated and hence, their history of syphilis infection remains unknown.

A piece of evidence or an idea that has been used to help support the Columbian hypothesis is the belief that the cure for a disease is often found at the starting place of that disease; “Our Lorde GOD would from whence this euill of the Poxe came, from thence would come the remedy for them” (Monardes 1577 cited by Crosby, 1969:221). In this case ‘holy wood’ or guaiacum was a substance used to help treat the disease and could only be found in the New World (Haiti) (Oriel, 1994; Parran, 1937; Quéral, 1990). As a result, many thought that this was proof of its origins from this part of the world. However in reality, holy wood was not a successful cure (Henschen, 1966) as it was no

more than a sudorific and laxative (Quétal, 1990). In fact, it was used for only a brief time with mercury proving more popular and effective.

The diametrically opposed pre-Columbian hypothesis contends that venereal syphilis had already been present in Europe for an extended period of time prior to the return of Columbus and his crew, but that it had been misdiagnosed as a number of other diseases, most importantly, leprosy (Cockburn, 1961; Hackett, 1963 and 1967; Holcomb, 1937). The supposed epidemic seen in the late 15th century was actually the clinical recognition of syphilis as a different disease entity from leprosy. As a result, increased numbers of cases began to be diagnosed as clinicians were educated on the differences between these two diseases.

Proponents of this hypothesis claim that venereal syphilis developed from bejel or yaws due to alterations in climate and living conditions (increase in hygiene and urbanization) as people migrated out of Africa around 10-15, 000 B.C. (Hackett 1963:26). However researchers are quick to point out that if treponemal disease was present in the Old World prior to 1492, written documentation and/or description of the disease would exist. Both Pusey (1933) and Wong (cited by Crosby, 1969) state that syphilis was not specifically mentioned by medical writers such as Galen, nor found in Chinese medical literature of antiquity, respectively. This lack of mention may not necessarily be true as Baker and Armelagos (1988) suggest that many of the original texts are too brief to provide concrete evidence of its presence or absence.

The third hypothesis called the Unitarian, asserts that syphilis has evolved and migrated with humans for centuries in both the Old and New Worlds as populations

altered their ecological and sociological environments (Hudson, 1963, 1965a and b, 1968). This theory suggests that the four syndromes (pinta, yaws, endemic syphilis [bejel] and venereal syphilis) caused by the treponemal bacteria are essentially the same infection but form a biological gradient with each of them manifesting in different environments. According to Hudson (1963, 1965a), treponematoses began as yaws in the hot, humid climates in sub-Saharan Africa and was transmitted by casual contact among scantily clad individuals. As people migrated to drier regions, the disease withdrew to the warmer, moister areas of the body (armpit, mouth and genital region) and became endemic syphilis. Then with the rise of urbanization and amelioration of personal and community hygiene, a new variant of the treponemal bacterium was selected in order to survive. Also, the disease could no longer be spread by casual contact as new barriers existed (e.g., washing with clean water and bathing with soap, separate dormitories and more clothes). Now the disease no longer affected immature individuals but instead had to rely on the close contact of mucous membranes of mature individuals, sexual contact and prostitution (generally more common in urban locales) to spread the now, venereal disease. Hudson (1965b) also suggests that both venereal and nonvenereal forms can exist in relatively close geographical proximities. For example, an urbanized area could have syphilis, but the surrounding rural region could have yaws/bejel due to their differences in hygiene and social milieus. It has even been suggested that venereal to nonvenereal conversions can occur (e.g., several studies have been reported by Willcox, 1974). According to Turner and Hollander (1957 and Hollander, 1981) temperature is an important factor in the pathogenesis of syphilis. They noted that when rabbits were

inoculated with treponemes and kept at cool air temperatures (20-35°C), higher infection rates, shorter incubation times and more severe lesions were observed than when maintaining them at higher air temperatures (37+°C). As a result, the difference in environments is then translated into the dispersal of the four different, but related, human pathogenic treponemal diseases: pinta and yaws are found in the tropics, endemic syphilis in the subtropics and venereal syphilis in the temperate zones.

The Unitarian and (most particularly) pre-Columbian hypotheses have a strong foothold in that they rely on an evolutionary framework using the forces of natural selection to alter the bacteria's existence and survival with a similarly evolving host. The fact that the four human pathogenic treponemes are morphologically identical and their pathogenesis is quite similar provides further support for these hypotheses. Earlier immunological studies also suggested that these diseases were similar as the immune system mounted relatively analogous responses and partial cross-immunity between the different syndromes had been recorded (Cates, 1998; Hardy, 1976; Knox et al., 1976; Plorde, 1994). However, recent research on guinea pigs suggests that yaws and syphilis have quite different pathogenic properties in that the former is dermatrophic while the latter is organotrophic (Wicher et al., 2000). Other noticeable differences include the character of the skin lesions, their onset and persistence.

With new technological advancements, genetics can now be used as evidence of a close relationship between the syndromes. Although only *T.p.* subsp. *pallidum* (venereal syphilis) has been completely sequenced (Fraser et al., 1998), individual genes from the other three (*T.p.* subsp. *endemicum* [endemic syphilis, bejel], *T.p.* subsp. *pertenue* [yaws],

and *T. carateum* [pinta]) treponemes have been compared. Comparisons show that they are quite similar genetically and should be placed within the same genus, but several polymorphisms (e.g., Cameron et al., 1999 and 2000; Centurion-Lara et al., 1998; Izard et al., 1999) exist to make them distinct enough to be separate species. If these differences point to distinct species, then the pre-Columbian hypothesis is likely correct. However, their true relationship will not be fully understood until all four human pathogenic treponemes are completely sequenced and compared to one another. For all we know the species designations that presently exist may have to be altered in the face of new molecular information.

All three hypotheses have several underlying qualities concerning the origins and spread of the pathogen. They all suggest that the disease in some form (either pinta or yaws) has had a very long history with humans and that it has spread throughout the world as a result of human migration. Where they differ at this point is in explaining the original precursor of the human pathogenic form. While Hackett (1963) (a pre-Columbian advocate) suggests a zoonotic origin, Hudson (1965a and b) (a Unitarian believer) and Cockburn (1961) (a pre-Columbian advocate) propose an evolution of a microorganism precursor but from different origins (free-living saprophytes vs. an ancestor in the *Spirochaetaceae* family, respectively). They also acknowledge the role of climate in influencing the different forms of the disease and the impact of urbanization on the development of venereal syphilis. In their simplest forms the Columbian, pre-Columbian and the Unitarian all understand the importance of human impact on a

pathogen, whether through migration or culture, and geography in the development of the treponemal bacteria.

2.2 The Beginnings of Syphilis

Soon after the syphilis epidemic began in the late 15th century medical practitioners became well-acquainted with the ulcerative and nodular characteristics of the disease, but it was not until the nineteenth century that information was gained concerning later stages of the disease and its effects on visceral, cardiovascular and neural areas (Oriol, 1994; Pusey, 1915). Although little was known about the causative agent at first, people were aware that it was transmitted via sexual relations. It was normally blamed on females in general, but prostitutes specifically, who supposedly harboured a “toxic substance” in their genital tracts and transmitted it to males by intercourse (Oriol, 1994; also mentioned by Cassel, 1987 and Quétal, 1990). This focus on blaming females is prevalent throughout the history of syphilis.

As stated earlier, syphilis had been placed in the category of leprous diseases, but once medical practitioners understood their differences, proper diagnosis and treatment ensued. However, misdiagnosis of syphilis would occur again throughout its history. For example, the study of syphilis was hindered in the 18th century because medical practitioners believed it to be one and the same as gonorrhoea. This was thought to be the case because the two diseases shared similar modes of transmission (sexual relations) and people were often infected with both pathogens at the same time. This confusion was compounded when a physician (Dr. John Hunter) tried to show that one could cause the other by inoculating a patient (thought to be himself) with exudate from what was thought

to be a gonorrheal case (Oriel, 1994; Parran, 1937; Pusey, 1915 and 1933; Quéral, 1990). Unfortunately the results pointed to their similarity as a single disease entity as Hunter had inoculated substances from a lesion of an individual who actually had been co-infected. This misconception was finally confronted years later by Benjamin Bell who pointed out the futility of doing only a single experiment where the two diseases were concerned. He suggested that to prove their identity, numerous experiments would have to be performed, that the symptoms and signs were quite different, and that mercury did not cure gonorrhea (Oriel, 1994).

Although the natural history of syphilis was generally known by the 19th century, it was Ricord (Oriel, 1994; Pusey, 1915) who proposed a simple viewpoint of its progression; that it started with a chancre (primary lesion), it disseminated throughout the body causing various signs and symptoms (secondary lesions) and finally it manifested into gummata (tertiary lesions) months to years after the primary inoculation. Also, the late 19th and early 20th centuries saw an increase in knowledge concerning the disease as different animals were used for experimentation because human inoculation was by then deemed unethical. Unfortunately human experimentation with syphilis did not end here*. For example, Neisser experimented on many different animals but his most comprehensive work on the disease was carried out on syphilitic monkeys. He was able to show that the bacteria were present in the blood soon after inoculation, excision of the

* The Tuskegee Syphilis Study was carried out from 1932-72 in Macon County, Alabama, U.S.A., as a medical experiment to learn more about the natural history of syphilis using 399 untreated African American men. Although much was learned about the clinical aspects of the progression of this disease, it has been overshadowed (rightly so) by the immoral and unethical practice of denying proper treatment to those with syphilis (United States Department of Health and Human Services, 2003).

chancre proved futile, secondary stages were contagious, and individuals with active syphilis could not be reinfected (Neisser 1911 cited by Oriel, 1994).

Over the years the cause of syphilis had been attributed to many agents including miasmas, toxic substances, and the like, but in 1905 the real causative agent was discovered by Fritz Schaudinn and Eric Hoffman (Oriel, 1994; Parran, 1937; Pusey, 1915 and 1933; Quéral, 1990). *Treponema pallidum* was first observed from a fresh preparation of a chancre under a microscope by Schaudinn and Hoffman who claimed to have observed pale spiral organisms they had never before seen that rotated longitudinally and underwent flexion. Unfortunately this form of visualization made it difficult to see the organisms, but when the dark field microscope was introduced in 1906 by Karl Landsteiner, this all changed. In fact according to Oriel (1994), research became very prolific with 750 papers appearing on this very subject by the end of 1906. Dark field microscopy is still used today to visualize the treponemes and help in the diagnosis of syphilis.

Since the beginning of the 20th century many other tests have been devised to help in the diagnosis of syphilis (see next chapter for more details). Many of these deal with the serological component (e.g., Wasserman reaction, Fluorescent Treponemal Antibody Absorption [FTA-ABS] test, Venereal Disease Research Laboratory [VDRL] test), but other methods also exist (e.g., Rabbit Infectivity tests [RIT], PCR) (Singh and Romanowski, 1999). By far the most sensitive and accurate tests that can be used at any stage of the disease are those which focus on DNA isolation via the polymerase chain reaction (PCR).

Throughout the history of syphilis many alternative methods have been used to treat the disease. Mercury was first prescribed for patients with syphilis as its cutaneous lesions looked similar to other skin diseases (e.g., scabies, psoriasis) which had been effectively treated with mercury in the past (Oriel, 1994; Parran, 1937; Pusey, 1915 and 1933). Mercury ointments were applied directly to lesions or the whole body and sometimes with additives to supposedly improve their action: aloe, sulphur, camphor, and sometimes exotic ingredients (viper's fat, earthworms fried in oil, frogs ground up alive) (Oriel, 1994). Mercury fumigations were also performed in hopes that people would sweat out the disease. Finally, mercury was also prescribed in oral forms. Although in some cases mercurial treatments were successful, they incurred a price: toxicity (and sometimes death from overdose) caused hair and tooth loss, abdominal pain, shaking, diarrhea, mouth ulcers and massive salivation. Another treatment that was preferred by syphilitics because it had no major side effects was that of guaiacum or holy wood (see page 15).

Mercury dominated the scene for most of the history of syphilis and it was really not until the beginning of the 20th century that new treatments were introduced. An arsenic-related compound called Salvarsan was introduced in the early 1900s which quickly diminished the signs of syphilis, but recurrences and side effects (e.g., dermatitis, jaundice and blood dyscrasias) plagued this treatment (Oriel, 1994; Pusey, 1915). All of the aforementioned treatments were good at alleviating early stages of syphilis, but useless for later stages. This situation was ameliorated by the use of malarial therapy (Oriel, 1994). Tertiary stage patients were purposely given malaria to create a febrile

environment that killed the treponemes and as soon as syphilis was cured, the malaria was terminated with quinine. The last important treatment prescribed to syphilitics was that of penicillin in 1943 which is still effective today (although cases have been cited where resistance has occurred [e.g., Klausner et al., 2004]). Penicillin chemotherapy has proven to be the best treatment yet for syphilis because it quickly cures with a short course of drugs, has minimal side effects and is effective for any stage of the disease.

2.3 The Pathogenesis and Pathologic Findings of Syphilis Infection

Essentially syphilis has three pathologic stages: primary, secondary and tertiary with each of these stages creating different clinical signs and/or symptoms. Only the most prominent manifestations are listed here with a more in depth discussion presented in the references cited below. The primary stage involves the presence of a single, painless, indurated ulcer called a chancre found at the site of inoculation with lymphadenopathy (Cates, 1998; Musher and Baughn, 1998; Plorde, 1994; Sanchez and Luger, 1993; Singh and Romanowski, 1999). This lasts several days to weeks and then disappears. Usually several weeks to months later, the secondary stage emerges as a full body copper coloured skin rash, alopecia, condylomata, cachexia, nausea and/or vague bone and joint pain (Cates, 1998; Musher and Baughn, 1998; Plorde, 1994; Sanchez and Luger, 1993; Singh and Romanowski, 1999). These secondary manifestations tend to disappear within weeks and anywhere from one year to 10-20 years later the final stage emerges. People who reach the tertiary stage of syphilis (not normally seen anymore because of chemotherapy) usually develop some form of cardiovascular problem (e.g., aortitis), a range of neurosyphilitic syndromes (e.g., meningeal, tabes dorsalis, paresis,

etc.) and gummata of the skin, bone and liver (Cates, 1998; Musher and Baughn, 1998; Sanchez and Luger, 1993; Singh and Romanowski, 1999). At any of these stages (with the first and second stages being more likely) a female infected with syphilis can pass the disease on to her fetus, causing congenital syphilis. Congenital syphilis produces a host of clinical signs such as persistent rhinitis, hepatomegaly, lymphadenopathy, gummata, osteochondritis, diaphyseal periostitis and osteomyelitis (Sanchez and Luger, 1993; Singh and Romanowski, 1999).

Syphilis is an intriguing disease because the bacteria can survive for extended periods (thus causing multiple stages) within the human body without initiating an immune response. Evasion of the immune system is not well understood but is most likely related to the low density of surface-exposed antigens. When compared with *E. coli* via freeze-fracture electron microscopy, it was observed that *Treponema pallidum* is relatively devoid of integral membrane proteins (Radolf et al., 1989). Without a sufficient number of integral membrane proteins there is little antigenic response. New research has also discovered that heterogeneous *tpr* (*Treponema pallidum* repeat) gene sequences exist for treponemes obtained directly from syphilis patients as well as those isolated from rabbit-propagated isolates (LaFond et al., 2003; Leader et al., 2003; Morgan et al., 2002). Heterogeneity of this transmembrane protein is believed to allow organisms to evade the immune response and consequently create reinfections and persistent infection.

It has also been proposed that the bacteria are able to survive for extended periods because they 'hide' out in areas that may not be as readily accessible by the immune

system (e.g., brain, eye, aorta and fetus) or undergo the degree of surveillance observed elsewhere in the body (Lukehart, 1992; Sanchez and Luger, 1993; Sell et al., 1980).

Baker-Zander and Sell (1980:401) state that the medium of the aorta is “rich in connective tissue and relatively inaccessible to circulating immune cells” and this same environment may also allow the organism to multiply and affect the brain or survive in gummata. As a result, the location of late granulomatous lesions (e.g., neurosyphilis, aortitis) may be attributed to the vascular system.

Syphilis is a systemic disease by the secondary stage, but it characteristically affects certain areas of the body more so than others. Although soft tissues and certain internal organs are also involved, skeletal distribution of the manifestations of syphilitic infection are discussed here because this is of greater importance to paleopathologists. Although not detailed until the next chapter, when syphilis affects the skeleton it predominantly creates lesions in the frontal bone (*caries sicca*), destroys nasal and palatal regions, causes vertebral and joint destruction and produces periostitis and osteomyelitis generally of long bones (e.g., *saber shins*). Involvement of the calvarium may be related to the location of numerous bacteria and T cell infiltrate. For example, Sell et al. (1980) have shown that *T. pallidum* and the T cell infiltrate tend to localize around hair follicles in the dermis causing perifollicular inflammation and they suggest that this may induce alopecia. By the tertiary stage, the T cell infiltrate may be so massive in this area that infection cannot be blocked, with the forehead gumma manifesting and leading to cranial involvement (and the creation of *caries sicca*).

It has been hypothesized that the characteristic distribution of periostitis and osteitis in syphilis (as well as in other diseases like pyogenic osteomyelitis) is directly related to the affected areas' increased susceptibility to injury (Jaffe, 1972; Kampmeier, 1964; Resnick and Niwayama, 1995; Steinbock, 1976; Williams, 1932). For example, the top of the skull and the tibia are more exposed to trauma than the humerus may be. Injury may compromise the area creating an environment conducive to bacterial invasion and/or hyper-response by the immune system. Jaffe (1972:925) states that lesion formation is predicated on "persistent low grade irritation", and not a single injury, that may be caused by "continued pressure or mild friction" in that particular area. I suggest that the fashion of wearing hats and wigs in the past may have provided 'continuous pressure', 'mild friction' or 'low grade irritation' conducive to the formation of forehead gummata and eventually caries sicca.

A predilection of spirochetes to lie dormant in bone has been suggested by Jaffe (1972), but without explanation. Bone is a relatively highly vascularized tissue with Haversian canals, Volkmann's canals and canaliculi servicing the circulatory system which can disseminate the bacteria into bone. However, there are areas (such as lacunae) which may become impeded due to their small size or possibly blocked off from the vascular system during bone remodeling or bacterial invasion and this is possibly where the spirochetes could lie dormant. In fact, Jaffe (1972) states that Schneider was able to visualize resting spirochetes in the lacunae of congenitally syphilitic infants, providing proof of their presence in bone.

Tissue tropism is also reliant upon the bacteria's ability to bind specifically or preferentially to certain surfaces via adhesin molecules which allow penetration of intercellular junctions and transport throughout the body (Wilks and Sissons, 1997). In vitro studies have shown that *Treponema pallidum* attach themselves to various mammalian molecules like fibronectin (Lee et al., 2003), laminin, collagen IV and I (Fitzgerald et al., 1984). Although fibronectin and collagen (specifically Type I) are associated with many other tissues and cells, they are also both connected to bone (Cotran et al., 1999) which may suggest the bacteria's ability to infiltrate and cause lesions in this area of the body.

A final and convincing proposal for the characteristic distribution of syphilitic pathogenesis has been related to the “anatomical relationship between the lymphatic and skeletal systems” (Buckley and Dias, 2002:183). These authors base their theory on the fact that the lymphatic system is integral to the pathogenesis of treponemal disease as the bacteria usually migrate and multiply in this area first before hematogenous dissemination. These authors illustrate that the treponemal lesions of the skeletal system are in direct relation to or in contact with lymph nodes and vessels that are not impeded by soft tissues (e.g., muscle attachments) and with pooling of lymph fluid (usually at extremities of limb bones) (see their Figures 2 and 3). They support their hypothesis by providing studies of other diseases (manifesting subperiosteal new bone growth) which show a direct relationship between lymph node infection and periostitis. For example, since the anterior region of the tibia is predominantly affected in treponemal disease, the association of the anterior tibial and popliteal nodes would provide the possibility of

tropism and an increased probability of affecting this bone element, respectively.

Although this theory is convincing it is problematic in that it cannot explain the presence of caries sicca because the calvarium region is not associated with any lymph nodes or vessels. Instead, the authors (Buckley and Dias, 2002:186) suggest that this area of the skeletal system may be affected by “metastatic seeding of spirochetes during the haematogenous spread” due to the highly vascular nature of the cranial vault.

As it stands, explaining the skeletal tropism and distribution of syphilitic infection may actually require a combination of several of the hypotheses presented above and only with more research will this be confirmed. However, modern cases of syphilis rarely progress to the stage where severe/full-blown bone lesions form and animal models fail to create skeletal lesions, thus making this area of study rather difficult.

2.4 The Social Environment of Individuals Used for Analysis

The samples used in this project come from 16 individuals from different geographical locales and time periods (more detailed information is available in section 4.1.1: pages 61-83). Nine samples come from individuals who lived in the United States of America during the time of the Civil War (1861-65) (MM2880-2, MM2880-4, 1002700, MM1154, MM1622, MM530, 295209 and MM2510); one from Belleville, Ontario living in the early to mid 1800s (B302); and six are from the United Kingdom dating before the voyage of Columbus (Whithorn gumma [AD 1300-1450], Gloucester Blackfriars SK77 [AD 1239-mid 15th century], Hull Magistrates Court [AD 1300-1450] HMC94-SK805, SK932, SK1121 and HMC94-SK1216) (see Figure 4.1 for geography).

Unfortunately for those samples collected during the Civil War time period (1861-65), little is known concerning their place of origin within the United States, although several of them are stated to have been soldiers. As a result, there is a heavy reliance on generalized ideas and concepts pertaining to military experiences with venereal disease during this time period.

Syphilis was a prevalent sexually transmitted disease by this time period with records from the medical history of Union troops in the Civil War stating that 82 per 100,000 cases were diagnosed during the war (Parran, 1937:74). One of our closest approximations regarding the prevalence of syphilis in the general public in the United States close to this same time period consists of a survey conducted in New York City in 1874 stating that approximately 1/19 (or 52 per 100,000) people suffered from syphilis (Kiple, 1993). As can be seen, syphilis was a considerable problem in the military and was considered more of a problem during times of war rather than peace (Beardsley and those references cited therein, 1976; Davidson, 1996; Spink, 1978), but this may have more do with bringing venereal disease discussions “out of the private sphere and into the center of public policy” to ensure a healthy armed force (Fee, 1988:123).

Lost time was considerable during the Civil War as syphilis disabled the fighting men and their training schedules (Ordranax, 1990; Steiner, 1977). Ordranax (1990:36) also states that if an individual “cannot withstand the duties and the hardships of a military life. He must be rejected.” Medical discharge based on complications associated with venereal diseases meant that the individual would not get paid (Beardsley, 1976), which was an important issue especially when their economic future was insecure

(Steiner, 1977). When put in this situation it is possible that soldiers would not report their infection* in order to remain in the army, thus increasing the probability of spreading the disease wherever they went.

Control measures during this time included diagnosis, hospitalization, provision of free treatment, reporting other infected soldiers and medical inspection, and the treatment and licensing of prostitutes (Bayne-Jones, 1968; Parran, 1937). However when placed in hospitals for treatment, many soldiers became co-infected with and often died from other acute diseases (e.g., typhoid, tuberculosis) (Steiner, 1977). Treatment was also an important factor in controlling syphilis, especially prior to discharge or disbanding, as it would help to decrease the spread of the disease to the soldiers' home towns/area. Treatment of prostitutes was included in the control measures because where large numbers of soldiers were, there were also a collection of prostitutes, especially when stationed near densely populated areas (Spink, 1978; Steiner, 1968).

Great attention and blame throughout history has repeatedly been placed on prostitutes as vehicles for venereal disease transmission (e.g., Baldwin, 1999; Brandt, 1987; Cassel, 1987; Fabricius, 1994). Prostitutes (and other women) suspected of having a venereal disease often had to succumb to mandatory treatments and rehabilitation programs as well as being centered out for example, by such policies as placing large placards on women's homes reading "suspected VD" during the First World War (Beardsley, 1976:196). This discrimination against women was based on the "traditional

* Neglecting to admit to infection or their history of syphilis was actually noted in one of the records of a sample from the Civil War period used in this project as Dr. Forwood states (1890) "The early history of this case [specimen 1002700] was difficult to obtain as he would not admit its specific origin; this would of course complicate any claim he might want to make for pension".

military attitude that men required sex to be good soldiers” (Exner and Fosdick cited by Brandt, 1987:54) and was often contrasted with the view that women were supposed to be the “embodiment of human virtues and the heart of the family” (Cassel, 1987:80). In order to diminish the use of prostitutes and divert soldiers’ attention, ‘good, clean fun’ activities, such as sporting events, theatre and educational programs (often centered on social hygiene) were instituted in the First World War (Beardsley, 1976; Brandt, 1987). So, importance was placed on trying to control as many variables as possible to limit the spread of the disease and its impact during times of war.

The individual from Belleville, Ontario was excavated from St. Thomas’ Anglican Church cemetery dated to AD 1821-74. Unfortunately no statistics were gathered to provide insight into the incidence or prevalence rates of syphilis in the 1800s in Canada, but it is recognized that it did exist during this time period (Cassel, 1987). Lack of records at this point in time suggests people either refused to seek medical treatment or the medical establishment refused to report cases of syphilis due to the moral undertones attached to having a sexually acquired disease. For example, Brandt (1987:17) states that doctors in the 19th century thought that revealing a patient’s venereal infection violated the tenets of the Hippocratic oath; also by labeling them a ‘carrier’, it would inflict irreparable harm; and by breaching confidentiality principles it might push patients to use other methods of treatment (most notably from ‘quacks’) to help conceal their disease. As a result, records pertaining to venereal disease reporting did not appear until after the First World War with most of the data pertaining to military personnel. At this point in time, it was estimated that 15.8% of the Canadian military with oversea assignments had

a venereal disease and that 4.5% of these cases were diagnosed as syphilis (Cassel, 1987:123).

Europeans emigrating mostly from Ireland, England and Scotland (and later the United States) were the original settlers of the Belleville community on the north shore of Lake Ontario's Bay of Quinte, which grew to become a major rural market centre by the early 19th century (Mika and Mika, 1986). The fact that Belleville was founded by European immigrants suggests that syphilis could have been transplanted to Belleville by its founding settlers. Migration of people and their concomitant infectious diseases is not an uncommon feature in the transmission of disease. The location of Belleville allowed for steamship accessibility facilitating trade of flour, lumber and wheat (and infectious disease). By 1856, Belleville was also connected by train to Montreal and Toronto (Mika and Mika, 1986) creating an even larger network of trade options and movement of people. Although Belleville was not an urban community by the mid 1800s, its connections to other destinations by both land and water routes and the draw of the market would ensure numerous contacts with transients and the opportunity for spreading disease.

The samples from the United Kingdom are from friary cemeteries of monastic towns and/or cities situated on water ways (e.g., ports) with Whithorn located on the west coast on the Isle of Whithorn of Galloway, Scotland (Cardy et al., 1997), Gloucester located on the river Severn near the southwest coast of England (Buchanan and Buchanan, 1980) and Hull being located on the river Hull on the east coast of England (Gillett and MacMahan, 1985) (see Figure 4.1 for more detail). These places were

bustling with commercial activities (e.g., importing and exporting raw and processed materials like iron, leather and wool) with a population to support such endeavors. As a result, all of the United Kingdom samples come from areas of relatively high populations (urban locales), ports and friary cemeteries. This environment presented an ideal situation for the spread of syphilis.

Statistics concerning the prevalence of syphilis in the United Kingdom prior to 1492 are nonexistent and as a result, rates have to be inferred from later records. According to records from the St. Bartholomew's Hospital that were examined by Pelling (1986), 21 out of 87 (24%) patients suffered from syphilis during 1547-8. In 1662 John Graunt published *Natural and Political Observations Made upon the Bills of Mortality* which states that 392 out of 229250 (0.17%) deaths could be accounted for by syphilis during the years 1629-39 and 1647-60 (cited by Fabricius, 1994:29-30). This is a relatively low rate as people were most likely dying from more acute diseases (e.g., tuberculosis, typhus).

Finding skeletonized remains of individuals with putative syphilis in friary cemeteries can be explained. Atkin (1993) suggests that the friars of Gloucester were possibly operating a hospital up until the late 15th century as this would have been the only establishment in the area which offered social services to the public. Timken-Zinkann (1969:355) also states that during a syphilis outbreak in Isenheim, Germany, in the 1490s, the Monastery of the Antonites accepted syphilitic patients to their hospital illustrating that they opened their doors to help all. Therefore, people with syphilis would

have most likely sought out the friars* for treatment. The possibility also exists that because syphilis was considered a punishment (Brandt, 1987; Cassel, 1987; Fabricius, 1994; Henschen, 1966; Parran, 1937), it would have been logical to spend the rest of one's time in or near monastic establishments in order to try to appease, make amends or repent sins. Placing moral judgments on those who were infected could be considered an effective way of increasing Christian following near the end of the 15th century.

Seeking help, treatment or refuge at a monastery was also a good way to segregate people with the disease in order to keep them out of the public's eye and decrease its spread. This has been attempted many times throughout the history of diseases (e.g., leper houses, tuberculosis sanatoriums, etc) with some success. It has been recorded that some places in Europe used drastic measures to try and stop the spread of the disease or quarantine people who had it. For example, Pusey (1915:11-12) writes that

On April 21, 1497, the town council of Aberdeen, Scotland, ordered that, for protection from the disease which had come out of France and strange parts, all light women desist from their vice and sin of venery and work their support, on pain, else, of being branded with a hot iron on their cheek and banished from the town. Six months later after the Aberdeen order, the Scottish Privy Council passed an edict ordering all inhabitants of Edinburgh afflicted with syphilis into banishment to the island of Inchkeith near Lear.

In 1496 Geneva also banned outsiders infected with syphilis from entering the city and those already within were not allowed to circulate (Registers of the Council of Geneva 1914 cited by Quéral, 1990). Quéral (1990) notes that destitute syphilitics, who had no place to go, usually ended up seeking refuge by the cathedral cloisters. A similar environment may have been present for those living in Gloucester, Whithorn and Hull.

* Of course it is also recognized that the clergy were not immune to this disease as Fabricius (1994:63-4) provides several citations to support the opposite.

Therefore, finding skeletonized individuals exhibiting syphilitic lesions in monastic cemeteries may represent examples of diseased individuals who sought refuge, health care and ‘normalcy’ (i.e., avoiding the stigma created by the common population).

Since syphilis has been termed a socially conditioned disease (Jankauskas and Urbanavičius, 1998), it tends to localize in populated areas, like cities. Such environments increase the probability of anonymous contacts which tends to increase the risk of contracting the disease and spreading it. Opportunities to disseminate syphilis would have been easy for those populations included in this study due to the movement of soldiers during the Civil War in the U.S.A. (possibly nearer to urban locales) and the movement of trains and ships to and from foreign destinations. In fact, the sea route is believed to be the culprit in bringing syphilis to India in the late 15th century (Quétal, 1990) and thus the role of seamen (and soldiers) in the spread of syphilis has had a long tradition (see also Fabricius, 1994). Higher prevalence rates in ports rather than inland cities have also been recorded in the recent past (Guthe and Willcox, 1954) which can be related to the increase in tourist traffic and sexual encounters among anonymous partners.

No matter what point in history is chosen, whether in the mid 1800s in North America or pre-1492 in the United Kingdom, similar ideas were held concerning syphilis: people feared it and feared those who had it. Grunpeck’s personal account of having this disease in 1503 exemplifies this point: after his illness was revealed to his friends, “they turned their backs on me as if some pursuing enemy had his sword at their throats, without giving a single thought to the obligations of human fellowship and friendship” (cited by Quétal, 1990:17). Social stigma is not just a thing of the past as people are

exposed to it even today (Lichtenstein, 2003). This moral judgment has impeded people from seeking treatment and in effect has probably allowed for syphilis persistence.

CHAPTER THREE:

IDENTIFICATION of SYPHILIS: SKELETAL, HISTOLOGICAL and MOLECULAR TECHNIQUES/APPROACHES

3.1 Skeletal Manifestations of Syphilis

Syphilis is a unique disease in that its symptoms wax and wane through three distinct stages when left untreated. It is usually assumed that skeletal lesions do not become manifest until the tertiary stage of syphilis. This is not always the case as many medical reports have observed skeletal involvement as early as the second stage. For example, Ollé-Goig et al. (1988) present two cases of individuals with secondary syphilis (confirmed by positive VDRL, FTA-ABS and RPR tests – see Table 3.1 for descriptions of these tests) with bone lesions. One patient presented with a “well-organised” periosteal reaction of the lower tibiae and “intracortical destructive lesions” of both tibiae and fibulae (*Ibid.*:199). The second patient had an “osteolytic lesion with an area of osteitis in the left frontoparietal area” (*Ibid.*:200). Many other cases have been reported in the literature whereby bone lesions have occurred in early stages of syphilis (Bauer and Caravati, 1967; Ehrlich and Kricun, 1976; Gomez Martinez et al., 2003; Gurland et al., 2001; Jaffe, 1972; Newman and Saunders, 1938; Reynolds and Wasserman, 1942; Shore, Kiesel and Bennet, 1977; Squires and Weiner, 1939; Thompson and Preston, 1952; Waugh, 1976; Wile and Sinear, 1916; Wile and Welton, 1940). Furthermore, according to Wile and Sinear (1916), a substantial number (36%) of their patients with primary and secondary syphilis had bone or joint invasion. Of course this latter report may be dubious due to limited knowledge and resources of this time period in medical history. According to Ollé-Goig et al. (1988:200), “the bone may be affected at all stages

of syphilis” which means that archaeological human remains with lesions indicative of this disease may not necessarily be in the tertiary stage, but may be in an earlier stage. Paleopathologists must keep this in mind when diagnosing this disease.

Venereal syphilis also has the ability to cross the placental barrier and cause congenital syphilis. The skeletal alterations indicative of this form of syphilis are not discussed here, as all specimens used were adults. In addition, the frequency of osseous congenital lesions is rare as evidenced by clinical studies from the past (Putkonen and Paatero, 1961; Wile and Sinear, 1916). As a result, the prevalence of observed congenital syphilis in the archaeological record is most definitely rare (e.g., Rothschild and Rothschild, 1997). This may be directly related to the infrequent preservation of fetal/infant remains in the burial environment in general. The possibility also exists that these individuals are missed in excavations because their remains are so small. It is also a known fact that many individuals infected with congenital syphilis die *in utero* or shortly thereafter. Wicher and Wicher (2001:355) state that approximately 50% will die *in utero*, be prematurely born, stillborn, or die shortly after delivery. This suggests that the preserved portions (i.e., skeletal tissues) from archaeological sites may not exhibit *any* changes that are characteristic of congenital syphilis because the duration of life of these children is too short for osseous alterations to occur. Also, the bony reactions are often remodeled during the child’s life, wiping away or altering any possible evidence of its presence (Jaffe, 1972:917). Jaffe also states (1972:921) that skeletal lesions that occur late in life due to congenital acquisition, “tend to resemble those of acquired syphilis in their appearance, their distribution and their entire nature”.

3.1.1 *Bony Responses Characteristic of Syphilitic Infection:*

When syphilis affects the skeletal system (usually in less than 20% of all cases), it does so in a relatively distinct manner; infection caused by syphilis engenders both destructive and proliferative osseous reactions. The bones most affected, in decreasing order, are the tibia, frontal and parietal, nasal-palatal area, sternum, clavicle, vertebrae, femur, fibula, humerus, ulna and the radius (Aufderheide and Rodríguez-Martín, 1998; Ortner and Putschar, 1985; Steinbock, 1976). Steinbock (1976:115) suggests that the prevalence of lesions on the tibia, clavicle, radius and ulna are the result of their greater exposure to irritation and minor trauma (also commented on by Resnick and Niwayama, 1995 and Jaffe, 1972). Bone alterations affecting these areas are caused by either chronic nongummatous (nongranulomatous) or gummatous (granulomatous) processes.

Nongummatous lesions are predominantly found in the long bones (but can also affect the nasal-palatal regions) and include periostitis (inflammation of the periosteum), osteitis (inflammation of the cortical bone) and a combination of the two (which occurs frequently when the infection is prolonged) generally referred to as osteoperiostitis. These types of lesions can occur in an isolated section of a bone or the whole bone may be involved.

Periostitic nongummatous lesions can be found on any bone but when they occur bilaterally, especially in the tibiae, they are considered pathognomonic of tertiary syphilis (Murray et al., 1990). The origin of nongranulomatous infection is most often in the periosteum with a sequential tendency to spread to the cortical bone and sometimes to the

medullary cavity. It is generally agreed that the proliferation of the periosteum is a gradual process of remodeling in which the newly built soft/fibrous bone is replaced by dense lamellar bone (Aufderheide and Rodríguez-Martín, 1998; Jaffe, 1972; Ortner and Putschar, 1985; Schultz, 2001; Steinbock, 1976). Nongranulomatous reactions occurring in the cortex cause cortical thickening with increased density and narrowing of the medullary cavity sometimes causing complete obliteration. This thickening is usually not uniform along the shaft and presents with irregular, roughened patches (Steinbock, 1976).

Gummatous lesions result in a centralized necrotic reaction due to a localized restriction of blood supply and increasing infection possibly due to increased levels of degenerative treponemal products within the area (Jaffe, 1972; Resnick and Niwayama, 1995; Steinbock, 1976). The necrotic cavity is then circumscribed by areas of hardened, plaque-like bone. The result is an irregular topography of bony destruction and proliferation. It is this particular osteosclerotic reaction that is considered characteristic of tertiary syphilis (Ortner and Putschar, 1985). Most gummatous lesions begin in the metaphysis with direct involvement of the medullary cavity, but can also occur less frequently with smaller lesions in the periosteum.

3.1.2 Location of Lesions:

3.1.2.1 Skull:

Lesions of the frontal and parietal bones, commonly referred to as Caries Sicca (first described by Virchow [1858, 1896]), are the most diagnostic features of syphilis

infection (Plates 4.3, 4.7, 4.8, 4.16, and 4.18; also see Figure 52 of Steinbock, 1976:131). These lesions are usually the result of gummatous osteoperiosteal infection most often originating in the frontal region but spreading to the adjacent parietal and facial bones as well (Ortner and Putschar, 1985:190). It is thought that caries sicca does not normally involve the inner table and will only destroy the outer table and diploë (Jaffe, 1972; Ortner and Putschar, 1985; Resnick and Niwayama, 1995) as a sequential extension of infection from the scalp (Steinbock, 1976:129). According to Jaffe (1972:930), it is not the gumma that erodes the bone, but rather the inflammation process *per se*. Jaffe (1972:932) also suggests that the actual disintegration of the outer table is influenced by “active vascularization of the diseased area, pressure from the gumma, and destruction of fragments of bone in the gummatous area.” If the disease is left untreated, the foci of caries sicca will heal leaving a “depressed, sclerotic, radially grooved stellate scar” surrounded by new necrotic areas (Ortner and Putschar, 1985:190) creating the appearance of irregular topography. Steinbock (1976:129) suggests that these lesions are in direct relation to small blood vessel pathways entering the cranium from the pericranium.

Caries sicca is created by a series of destructive and regenerative bone processes. It begins with erosion of the outer table of the calvarium that extends to the diploë causing what Hackett calls “serpiginous cavitation” (See Figure 2 in Hackett, 1975:233). On appearance, this causes generally windy, snake-like trails in the bone. As this is proceeding, bone formation on the circumference of the lesions occurs creating “nodular cavitation”. Next, bone remodeling of the area results in a sclerotic, smooth appearance

of bone that is eventually resorbed creating a stellate, wrinkled lesion (refer to Figure 52 in Steinbock, 1976:131). Steinbock (1976:131) refers to the overall appearance of caries sicca as “worm eaten”. According to Schultz (2001), this outline of macroscopic bony changes is in direct relation to what can be visualized by microscopy (e.g., the author believes that Schultz is referring to the manifestation/visualization of Polsters at the microscopic level – see discussions on page 48-9).

The nasal area can also be affected in later stages of syphilis by destruction of the tissues at the base of the nose causing this area to sag/sink (often referred to as saddle nose) (Jaffe, 1972; Ortner and Putschar, 1985). It is thought that lesions in this area are most often the extension of syphilitic ulcers from the nasal mucosa (Jaffe, 1972). The thin bones of the nasal and palatal regions are often destroyed and perforated creating an “enlarged and empty cavity in the dry skull” bordered by sclerotic bone (Ortner and Putschar, 1985:192).

3.1.2.2 Long Bones:

The tibia is the long bone most involved in syphilitic infections. Bony reaction caused by nongummatous lesions results in the build-up of periosteal (lamellar bone and plaque-like exostoses) and cortical regions (Aufderheide and Rodríguez-Martín, 1998; Ortner and Putschar, 1985). Excess osseous production along the shaft of the tibia creates a heavy, thick bone with a characteristic shape (curved on the anterior and flattened on the posterior), known as a saber shin (see Steinbock, 1976:105). Long bones can also be affected by gummata (cloacae) which form a scooped-out depression starting at the periosteum and continuing into the cortex. The cloacal openings tend to be small focal

areas of destruction often grouped with rough, thin margins. Those gummata that manage to penetrate the medullary cavity are usually larger in size bordered by “perifocal reactive sclerosis” (Ortner and Putschar, 1985:197). The reactive, sclerotic bone found at the margins of these gummata is thought to reflect a local reaction to the presence of necrotic, gummatous bone (Aufderheide and Rodríguez-Martín, 1998). Focal areas of destruction in long bones create a weakened structure often resulting in fractures.

3.1.2.3 Joints:

Syphilis can also affect the larger joints (e.g., knee, shoulder and elbow) via a gummatous arthritis (destructive in nature), but this condition is uncommon and often leaves no visible alterations in skeletonized remains. However when present (in the form of osteophytes, marginal lipping and eburnation), it occurs unilaterally and lesions are hard to discriminate from degenerative arthritis (Aufderheide and Rodríguez-Martín, 1998; Ortner and Putschar, 1985; Steinbock, 1976).

Joint involvement can also be a result of neurosyphilis, especially during tabes dorsalis of the tertiary stage when the spirochete enters the cerebral spinal fluid. Syphilis causes damage to the sensory nerves in the dorsal roots which can impair joint position sense and pain sensation (Cotran et al., 1999). Joint destruction is the result of repeated and excessive trauma (known as Charcot’s Joint) which may allow for bacterial invasion and a superimposed infection (Aufderheide and Rodríguez-Martín, 1998).

3.1.2.4 Spine:

Involvement of the spine is a confusing matter. According to Ortner and Putschar (1985:197-8), Steinbock (1976:126) and Jaffe (1972:938), the cervical spine is affected

more often than any other vertebral section possibly due to an extension of nasopharyngeal mucosal lesions. Steinbock (1976) reports that both necrotic and proliferative bony reactions affect the periosteum of the vertebral body creating an abnormal shape or size. However, Aufderheide and Rodríguez-Martín (1998:161) state that it is the lumbar region that is affected more often by degenerative changes, sclerosis and malalignment. At any rate, in some circumstances the vertebral column can be involved during syphilitic infection of the skeletal system.

It must be noted that all of the preceding skeletal manifestations of acquired venereal syphilis are not always found in every individual who has this infectious disease. In addition, not all of these manifestations will present themselves in the classic chronological order that has become characteristic to the clinical identification (see earlier discussion on osseous alterations observed in secondary syphilis cases).

3.1.3 *Differential Diagnosis*

When making a diagnosis of syphilis in skeletal remains, several other diseases must be considered due to their similarity in manifestations. Two infectious diseases critically important in this respect are those that are most closely related genetically to syphilis: Yaws (*T.p. subsp. pertenue*) and Endemic Syphilis or Bejel (*T.p. subsp. endemicum*). These two diseases are so similar skeletally (i.e., no lesion seen in venereal syphilis is not observed in the other two) to syphilis that it has been said that their only differences lies in the quantity of lesions (Steinbock, 1976:143). For example, skeletal involvement is reportedly approximately 1-5% for both yaws and bejel, but 10-20% for

syphilis (Aufderheide and Rodríguez-Martín, 1998:156-8; Steinbock, 1976). Although syphilis can affect the nasal-palatal region, this is said to be more characteristic of yaws and bejel (Aufderheide and Rodríguez-Martín, 1998; Ortner and Putschar, 1985). Nasal and maxillary destruction can cause a condition known as gangosa and in severe cases, the destruction can spread to adjoining bones creating a large crater in the middle of the face. In both yaws and bejel, saber shin and nasal-palatal destruction are frequently observed, which is shared with syphilis, but all other lesions are rarely found in these nonvenereal diseases. The frontal bone can be affected in yaws, but the lesions are shallow and pitted unlike the deep, destructive and proliferative lesions characteristic of caries sicca observed in venereal syphilis.

A major difference between the onset of shared skeletal lesions is that syphilis usually manifests later in life, unlike yaws which is a childhood disease. Another distinction between syphilis and yaws is that in the latter, dactylitis (“subperiosteal bone apposition parallel to the cortex with resorption of the original cortex” [Aufderheide and Rodríguez-Martín, 1998:156]) is observed more frequently in several bones of subadults (Steinbock, 1976). Although both syphilis and yaws have joint destruction, their difference lies in the fact that in yaws, reparative bone can be found on the joint surface (Ortner cited by Aufderheide and Rodríguez-Martín, 1998).

Apart from the difficulty in deciphering the different treponematoses that affect bone, several other diseases have very similar manifestations to syphilis. For example, various tumors (e.g., primary osteogenic sarcoma, meningioma, metastatic carcinoma and multiple myeloma), other infections (e.g., tuberculosis, leprosy and pyogenic

osteomyelitis) and Paget's disease must all be considered in the differential diagnoses. Although these diseases may resemble syphilis, they all have bony distinctions that can be used to differentiate them. For example, both metastatic carcinoma and multiple myeloma can be differentiated from syphilis because their lesions are smaller and purely lytic in nature, with very little bone rejuvenation (Aufderheide and Rodríguez-Martin, 1998; Steinbock, 1976). A main difference between tuberculosis and syphilis is that in the former disease cranial lesions, if they occur, begin on the inner table (compare with syphilis destroying the outer table first) (Ortner and Putschar, 1985). In addition, tubercular lesions are mainly destructive in comparison to the mixture of osteoclastic and osteoblastic reactions seen in syphilis. Also, when the nasal-palatal region is perforated it can be distinguished from leprosy because in syphilis the frontal bone is also involved and accompanied by sclerotic response bordering the lesion (Ortner and Putschar, 1985). Finally, Paget's disease creates a totally different histological picture than syphilis (mosaic structures compared to distinct formations seen in syphilis – see below).

3.2 Histological Characteristics of Syphilis

Microscopic analysis of bone is another tool researchers use to aid in the study of disease in ancient human populations. The normal micro-architecture of bone and effects caused by diagenesis within the burial environment are important aspects to understand in order to make a proper diagnosis of the presence of pathology. Normal micro-architecture of bone consists of a lamellar distribution of collagen fibres situated around osteons/Haversian systems, between Haversian systems (interstitial lamellae) and also in periosteal (external circumferential) and endosteal (internal circumferential) locations.

The orientation of the collagen fibres in mature bone alternates from one layer to another producing a pattern of birefringence of the concentric lamellae known as the Maltese cross of Haversian systems under polarized light (Garland, 1989; Schultz, 1997) (Plate 3.1).



Plate 3.1: Illustration of ‘normal’ bone histology Cross section of cortical bone of the humerus of a modern cadaver Photo courtesy of Patrick Beauchesne.

Diagenesis is an important aspect to consider when analyzing archaeological bone because in some cases the systemic environmental degradation of histological structures can cause pseudopathology (Wells, 1967). Diagenesis can be described as a complex of changes caused by chemical, biological and physical processes acting on a specimen while buried in different types of environments. For example, chemical elements in the burial environment can eat away at bone to make it appear as if osteoclastic processes are at work. Only by understanding the different presentation of diagenetic alterations of histological structures, normal micro-architecture and pathological manifestations can a more precise diagnosis be made.

Information pertaining to histological alterations of the skeletal matrix caused by syphilitic infection has really only been recently approached by one individual: M. Schultz (1994; 2001; 2003). His work is based on looking at bones labeled “probably syphilis” and/or presenting macroscopic and radiological criteria characteristic of venereal syphilis. According to Schultz (1994; 2001; 2003), criteria do exist for deciphering venereal syphilis from other specific and non-specific inflammatory diseases when using light microscopy of ground thin sections of bone viewed under polarized light.

Schultz (1994) states that the periosteal thickening of long bone shafts and the concomitant microscopic changes seen in venereal syphilis are a regular occurrence most noted when the process of healing and remodeling of compact bone has not yet finished. The first criterion that Schultz presents is the presence of a line or band-like structure (“Grenzstreifen”; translated as “border stripes”) which separates the primary/original cortical bone from the newly active periosteal layer laid down during bouts of infection (see Figure 1 of Schultz, 1994:66; Schultz, 2001:106; Figure 6.19 of Schultz, 2003:86). The next criterion is called the “Polster” (translated as “padding”). This structure represents pillow-like, polyp-like or villous proliferations of very dense parallel lamellae found in the highly thickened periosteal layer of cortical bone in the shaft of a long bone (see Figure 2 of Schultz, 1994:67; Schultz, 2001:107; Figures 6.18 and 6.19 of Schultz, 2003:86). He also suggests that syphilitic alterations of the microstructure of bone also present with ‘sinous’ resorption lacunae between the original bone surface and the newly

calcified layers, but warns that these structures are also found in other non-specific inflammatory diseases (Schultz, 1994:65).

Unfortunately, with recent microscopic approaches to other infectious diseases, it is evident that some of these structures “indicative” of syphilis are also present in other diseases. For example, Schultz and Roberts (2001) have observed the presence of Polster-like and Grenzstreifen-like structures in histological thin sections of leprosy bones. To differentiate, Schultz (2001:126) states that leprosy Polsters are “rudimentarily developed and relatively flat” (see Figure 8E in Schultz, 2001:127) in comparison to syphilitic Polsters (larger and more pillow-like with organized lamellae). The Grenzstreifen-like structures in leprosy appear to be thinner (less lamellae) and will not show alterations in the subperiosteal bone nor the endosteal bone or trabeculae (no osteoclastic changes in both) (Schultz, 2001:128). Grenzstreifen-like structures can also be found in nonspecific inflammatory diseases (e.g., hematogenous osteomyelitis), but can be differentiated via their manner of production: syphilitic Grenzstreifen are band-like structures laid down in a very slow manner creating a solid mass of newly built bone versus an aggressive, rapidly laid down and often disorganized (combination of newly built bone with old bone structures) structure of bone in nonspecific inflammatory diseases (Schultz, 2001). Grenzstreifen can also be observed in microscopic analyses of skeletal remains suffering from endemic syphilis. This discussion shows that there is a fine line between diagnosing syphilis and other infectious or inflammatory diseases, as well as illustrating the limited range of osseous reactions.

3.3 Molecular Diagnosis of *Treponema pallidum* subspecies *pallidum*

Laboratory diagnosis is an important confirmatory tool for clinicians because several infectious diseases (e.g. gonorrhea, chancroid) manifest in a similar fashion to syphilis. In response to this, many different methods exist to aid in the identification of syphilis in clinical settings. A summary of these methods, how and when they are used and the advantages and disadvantages are presented in Table 3.1.

Two important disadvantages exist for many of these methods (e.g., Dark-field, DFA test, Non-Treponemal and Treponemal tests). These are the inability to distinguish between *T. pallidum* subsp. *pallidum* and other human pathogenic treponemes and test sensitivity usually decreases as the disease progresses (e.g., see Table 3.1 of Van Dyck et al., 1999:41 and Table II of Wicher et al., 1999:1037). As well, serologic methods are relatively insensitive during the early stages of infection and antibody detection is only possible after the infection has progressed 1-3 weeks after the presentation of the chancre (Larsen et al., 1995; Larsen et al., 1999). PCR is the only method that has the ability to differentiate between treponemal species and has the sensitivity to detect approximately 10 organisms or less (Wicher et al., 1999:1035). An even more sensitive method is reverse transcriptase PCR (RT-PCR) which allows for the detection of a single treponeme (Centurion-Lara et al., 1997a). The major disadvantage of PCR methods in identifying syphilis in clinical samples is that they are unable to recognize present versus past infections (i.e., live vs. dead organisms). However, this can also be said of the other diagnostic methods (Van Dyck et al., 1999).

Method	How it is Used	When it is Used	Advantage	Disadvantage
Dark-Field Microscopy	Look for characteristic spiral shape and motility/movement of treponeme from exudate of a lesion	Primary and secondary lesions; congenital	Instant and direct diagnosis; detects syphilis prior to Ab production	Well-trained and experienced personnel needed; examination must be done promptly; sensitivity ~70%
Direct Fluorescent Antibody (DFA) test	Identifies organism via fluorescent isothiocyanate (FITC)-labeled anti <i>T. pallidum</i> Ab. from collected exudate	Primary and secondary lesions; congenital	Motile organisms not required; can detect organisms in both exudates and formalin-fixed tissues	Sensitivity slightly better than Dark-Field
Non-Treponemal tests (serological): e.g., VDRL, RPR-card	Immune flocculation using cardiolipin, lecithin and cholesterol as Ag	All stages; used more as a screening process in conjunction with Treponemal tests	Inexpensive	Nonspecific; false-positive reactions frequent with autoimmune disorders
Treponemal tests (serological): e.g., FTA-Abs, MHA-TP, IgM Abs	Direct detection of Ab to <i>T. pallidum</i>	All stages; congenital	More specific than Non-treponemal tests; FTA-Abs test most specific in this category, but MHA-TP easiest to perform and can be done in "batches"	MHA-TP not very sensitive to primary syphilis
Rabbit Infectivity Test (RIT)	Suspected clinical sample injected into rabbit testes; look for presentation of lesions on rabbit	All stages; congenital	Direct visual observation of disease (chancres/lesions); almost as sensitive as PCR	Long incubation time and use of animals
PCR	Amplification of treponemal sequence specific DNA	All stages including after treatment; congenital	Some tests can differentiate between pathogenic and nonpathogenic species of treponeme as well as other genital ulcer diseases	Cost; lack of standardized methods

Table 3.1: Summary of Diagnostic Laboratory Methods for Syphilis.

VDRL, Venereal Disease Research Laboratory; RPR, rapid plasma reagin; Ag, antigen; Ab, antibody; FTA-Abs, fluorescent treponemal antibody-absorption; MHA-TP, microhemagglutination assay for antibodies to Tp; ELISA, enzyme-linked immunosorbent assays (Summarized from Luger, 1988; Plorde, 1994; Singh and Romanowski, 1999; Van Dyck et al., 1999; Wicher et al., 1999).

The molecular identification of *T.p.* subsp. *pallidum* is now possible because its genome has been completely sequenced (Fraser et al., 1998). Genome analysis reveals that it is a circular chromosome consisting of 1,138,006 base pairs (making it one of the smallest prokaryotic genomes) with an average G + C content of 52.8%. Unfortunately, the other pathogenic treponemes (*T.p.* subsp. *endemicum* [endemic syphilis or bejel], *T.p.* subsp. *pertenue* [yaws] and *T. carateum* [pinta]) have not been fully sequenced. For now, genetic differences among a select number of genes/proteins (mostly single-copy genes) from these species are instead the subject of comparisons between the human pathogenic treponemes. When the genomes of these treponemes have been fully sequenced, the possibility exists that a better understanding of these species and their evolution will be achieved.

The first reported experiments of treponemal DNA isolation by PCR methodologies were performed by Noordhoek et al. (1990) and Hay et al. (1990a). The former group isolated DNA from two homologous genes in syphilis (*tpf-1*) and yaws (*tyf-1*) and discovered a base-pair difference (A-G) between these subspecies. The latter group was also able to amplify DNA from the *tmpA* gene. Since then many genes have been targeted by PCR: DNA polymerase I gene (Liu et al., 2001; Marfin et al., 2001), the BMP gene (Noordhoek et al., 1991; Wicher et al., 1992), 47-kDa protein gene (Burstain et al., 1991), 16S rRNA gene (Centurion-Lara et al., 1996), 15-kDa lipoprotein gene (Centurion-Lara et al., 1997b) and the Tp92 gene (Cameron et al., 2000). Many of these genes have been poorly characterized and their functions remain unknown. Also, few of these genes show differences among the human pathogenic treponemal subspecies.

PCR has also been used to help differentiate between several diseases that cause genital ulcers (Orle et al., 1996). Orle et al. (1996) set up an assay to simultaneously amplify (known as a multiplex PCR) DNA from *Haemophilus ducreyi* (causative agent of chancroid), *T. pallidum* and herpes simplex virus types 1 and 2 from clinical samples. In one reaction this test provides genetic evidence of the presence of one of these diseases. PCR has also been used to isolate syphilitic DNA in individuals co-suffering with HIV (Hay et al., 1990b; Horowitz et al., 1994). These examples provide proof that PCR is an important methodology for differentiating between several diseases with similar clinical presentations in a quick and efficient manner.

Not only have PCR methodologies been able to differentiate between different diseases, but they have also encountered intra-strain heterogeneity in the DNA polymerase I gene in Arizona (Sutton et al., 2001) and the *arp* and *tpr* genes in South Africa (Pillay et al., 1998; Pillay et al., 2002) within the *T.p.* subsp. *pallidum* genome. This form of molecular typing revealed that although predominant subtypes existed, strain population was still very diverse and varied geographically (Pillay et al., 2002). Another area of heterogeneity lies in a surface-exposed molecule called the *TprK* (*T. pallidum* repeat) gene. Centurion-Lara et al. (2000) and Stamm and Bergen (2000) discovered multiple sequences in three Nichols strain isolates (Sea 81-4, Bal 7 and Bal 73-1) and Nichols strain UNC and Street Strain 14, respectively. It was suggested by these authors that the variable repeats found in the *TprK* gene may be involved in antigenic heterogeneity and possibly evasion of the immune system (also confirmed by Morgan et al., 2002 and LaFond et al., 2003). It is also believed that these variants represent the

existence of multiple subpopulations (Centurion-Lara et al., 2000) and not the presence of multiple copies within a single organism (Stamm and Bergen, 2000).

Several PCR methodologies have also been successful in identifying syphilis from various types of samples taken from syphilitic individuals. *T. pallidum* DNA has been detected by PCR in genital ulcers, blood, brain and heart (i.e., adventitia) tissue, cerebral spinal fluid (CSF), amniotic fluid, serum samples and even paraffin-embedded tissue (Burstain et al., 1991; Grimprel et al., 1991; Hay et al., 1990a and 1990b; Horowitz et al., 1994; Jethwa et al., 1995; Noordhoek et al., 1991; O'Regan et al., 2002; Sutton et al., 2001; Wicher et al., 1992; Zoechling et al., 1997). In addition, PCR has the ability to detect treponemal DNA in cases of congenital syphilis. Amniotic fluid, fetal and neonatal sera and CSF were tested for treponemal invasion with similar success as that seen with rabbit infectivity testing (Grimprel et al., 1991; Hollier et al., 2001; Michelow et al., 2002; Nathan et al., 1997; Sánchez et al., 1993). What is even more interesting about these studies is that in many cases the investigators were able to isolate syphilitic DNA from some of these samples even when the expectant mother was in the early latent stage. The success of treponemal DNA amplification from variable sources provides proof that the disease disseminates throughout the body. Successful detection of syphilitic DNA from samples taken from later stages suggests that this disease is quite persistent and organisms remain in the body for quite some time. These results suggest the hypothesis that bone (with lesions created in later stages) may be a good source from which to amplify treponemal DNA.

Two areas of the treponemal genome, 15-kDa Lipoprotein and the Glycerophosphodiester Phosphodiesterase (GPD) genes, have been chosen for this project because of a recognized base-pair difference specific to *T.p.* subsp. *pallidum* that has not been found in any of the other tested human pathogenic and nonpathogenic treponemes. This of course cannot be said for any of the other PCR assays discussed above. The 15-kDa lipoprotein is a pathogen-specific membrane immunogen (possibly single copy) (Purcell et al., 1989) while GPD is an outer membrane protein (a potential immunoprotective antigen) that exhibits enzymatic activity during phosphodiester metabolism (Cameron et al., 1998).

According to Centurion-Lara et al. (1998) and Cameron et al. (1999) *T.p.* subsp. *pallidum* organisms differ from other human nonvenereal pathogenic treponematoses by at least one base pair in these two different genes. The base pair differences referred to are at position –150 of the 15-kDa lipoprotein gene (C>T for non-pallidum subspecies observed by Centurion-Lara et al. [1998]) and position 579 of the GPD gene open reading frame (an A>G for other human nonsyphilis subspecies observed by Cameron et al. [1999]). Due to the fact that nonsyphilis forms of treponematoses differ from human venereal syphilis by only one nucleotide in certain areas provides an ideal situation for the production of an ARMS test to help differentiate the treponemal subspecies.

The acronym ARMS refers to *Amplification Refractory Mutation System*. This system is a modification of the polymerase chain reaction used to detect single point mutations or heterozygotes (Newton et al., 1989; Strachan and Read, 1999). It is sometimes referred to as allele-specific PCR. This technique uses primers that are

identical to a DNA sequence with the exception of the extreme terminal 3' nucleotide: the nucleotide difference of the 'mutant'. The 3' terminus is used in this manner because the DNA synthesis step in PCR is dependent on correct base-pairing at this end (Strachan and Read, 1999:127). If the specific base-pair is not encountered, binding and extension of the oligonucleotide is inhibited (Figure 3.1) and hence, no identification of the heterozygote/mutant.

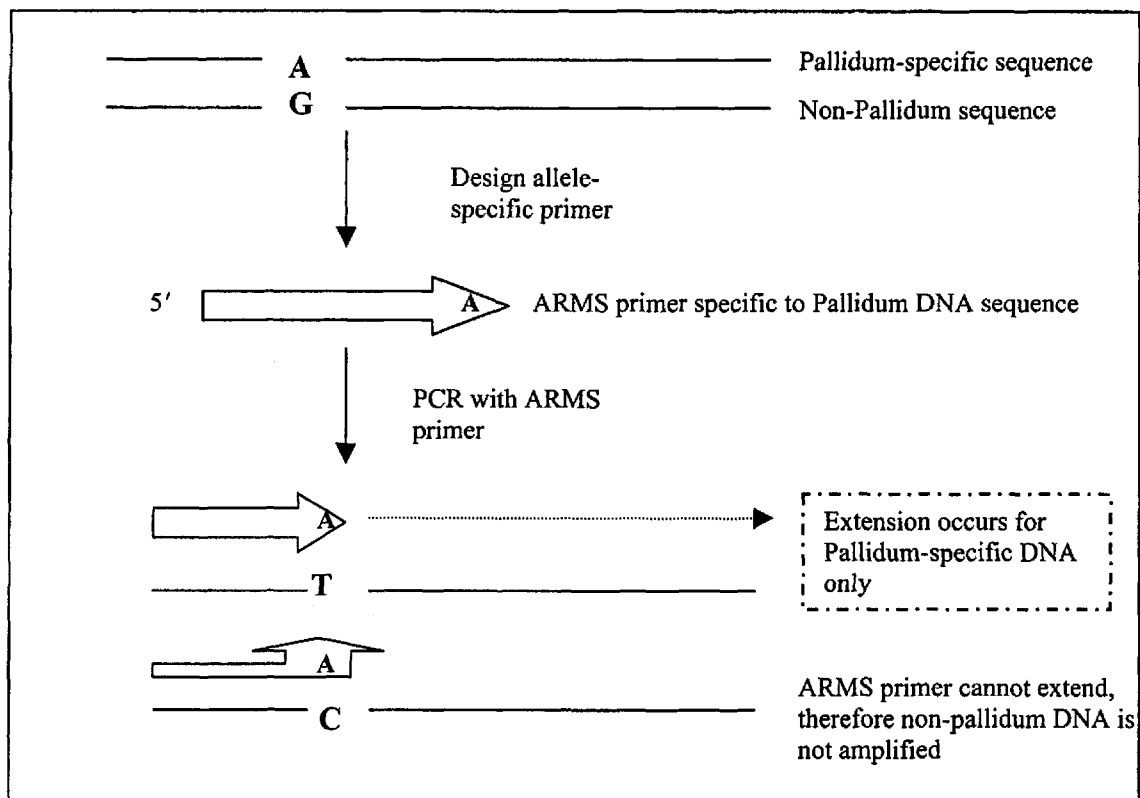


Figure 3.1: Diagram illustrating how an ARMS system would work for the GPD gene.

Some ARMS systems also purposely alter another base-pair near the 3' terminus to ensure (or increase the specificity) that amplification will only occur with the 'mutant' DNA as some cases have shown the amplification of 'normal' DNA when only one

mismatched base appears in the primer (Newton et al., 1989). This method of PCR has proven to be very quick and reliable in identifying point mutations of certain diseases (e.g., β thalassaemia [Old et al., 1990]) as no sequencing or any other manipulation (e.g., restriction digests) is required of the PCR results. It has even been used* in ancient DNA circumstances to isolate the HbS mutation (sickle cell) in predynastic Egyptian mummies exhibiting possible bone markings caused by hemoglobinopathies and thalassemias that are often associated with malarial infection in this part of the world (Marin et al., 1999).

Most of the macroscopic and microscopic characteristics of syphilis provide a good basis for its identification in ancient human remains. However, syphilis has the ability to mimic many other diseases which creates problems in confident diagnoses of the presence of this infectious disease. The recent sequencing of the *T.p.* subsp. *pallidum* genome and several genes of other human pathogenic and nonpathogenic treponemes create the opportunity to develop a molecular system of identification and thus, a more concrete diagnosis of the presence of syphilis in archaeological remains.

* This study was successful in that a PCR product was obtained in three out of ten samples, but they have not been sequenced to verify the mutation.

CHAPTER FOUR: MATERIALS and METHODS

Bone and/or tooth samples from sixteen different individuals from different locales were utilized in this study (see Figure 4.1a and b). Near the beginning of this project it was realized that another group had already successfully developed a molecular technique to identify syphilis in ancient human remains. Kolman and colleagues (1999) described a single-base mutation distinguishing *T.p.* subsp. *pallidum* from four other human and nonhuman treponemes (*T.p.* subsp. *endemicum*, *T.p.* subsp. *pertenue* and *T. carateum*) in the 5' untranslated region of the 15-kDa lipoprotein (tpp15) (Centurion-Lara et al., 1998). Their procedure requires sequencing and restriction enzyme (RFLP) analyses. The Kolman et al. (1999) procedure was tested on clinical and sixteen archaeological specimens, but I thought that perhaps a faster and cheaper method could be developed which would eliminate both RFLP and sequence analyses.

By using an ARMS test in this project, these two methods would not be needed as oligonucleotides are developed specifically to detect a nucleotide polymorphism present in the different treponemal pathogens. This means that just the mere presence of a band in the right location of a polyacrylamide gel provides proof that the right sequence (i.e., from *T.p.* subsp. *pallidum*) has been amplified.

Two other molecular tests were also used in this project to isolate treponemal DNA from the samples. Even though the amplicon for the polymerase I gene (*polA*; Marfin et al. [2001] and Liu et al. [2001]) is 377 bp, it was chosen for this project because of its robustness and sensitivity (detection limit is 10-25 organisms via gel and a single

organism using ABI 310 Prism Genetic Analyzer) as well as its ability to isolate treponemal DNA from latent cases of syphilis. The *polA* gene system has been tested on an extended variety of organisms (59 spirochete and nonspirochete, as well as *T.p.* subsp. *pallidum* organisms) with only *Treponema pallidum* subspecies (i.e., subspecies *pallidum*, *pertenue* and *endemicum*) being positive via PCR (Liu et al., 2001).

The second gene of interest, the 47-kDa membrane protein with penicillin binding activity (Deka et al., 2002), was chosen for this project because of its proven success in isolating treponemal DNA from different clinical samples (e.g., Burstain et al., 1991; Grimprel et al., 1991; Jethwa et al., 1995; Moskophidis and Peters, 1996; Nathan et al., 1997; Sánchez et al., 1993). Another advantage centers on the fact that PCR amplification of this membrane protein gene has also been used on archival material. Zochling et al. (1997) used a nested PCR system (with final amplicon of 196bp) to isolate treponemal DNA from paraffin embedded tissue archived from 1966 to 1981 (even from those samples diagnosed with tertiary syphilis).

Before a molecular test for syphilis should be used on ancient human remains, macroscopic lesions indicative of this disease should be diagnosed first. The most relevant skeletal indicators used in this project were the presence of osteomyelitis, periostitis and osteitis as well as gummatous lesions in specific areas (e.g., tibia [causing saber shin], nasal cavity) and, most importantly, caries sicca of the cranial vault. Unfortunately some of these changes are also characteristic of other diseases (e.g., tuberculosis, osteosarcoma, pyogenic osteomyelitis and Paget's disease). To help discern if the macroscopic alterations are caused by syphilis, histological techniques can also be

employed. The presence of ‘Grenzstreifen’ and ‘Polsters’ with ‘Achatknochen’ (i.e., dense lamellae) within thin sections of pathological bone can provide evidence of the presence of venereal syphilis. Histological sections were taken from several individuals to help in the identification. In addition, this method was employed to assess the state of preservation of the bone.

4.1 Skeletal Analyses

Samples for this project were chosen primarily on the basis of appropriate descriptions found in historic medical documents and the presence of skeletal alterations characteristic of syphilis in archaeological specimens. An in depth discussion of the osseous changes induced by the presence of the syphilis spirochete is presented in the previous chapter (see pages 38-47) but a summary of the predominant forms of pathology is provided in Table 4.1. As with any paleopathological approach to the study of human remains, differential diagnoses were also considered.

Characteristic Bone Changes in Syphilis
Caries Sicca
Gummatous Osteomyelitis
Osteitis
Periostitis
Saber Shin
Nasal Destruction
Vertebral and Joint Destruction

Table 4.1: Summary of skeletal lesions indicative of syphilis.

4.1.1 *Site (where applicable) and Sample Descriptions Pertaining to Pathology:*

Sixteen individuals sampled (refer to Table 4.2 for complete list) for this project originate from various locations around the world and from various time periods (see

Figure 4.1a and b). They 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.

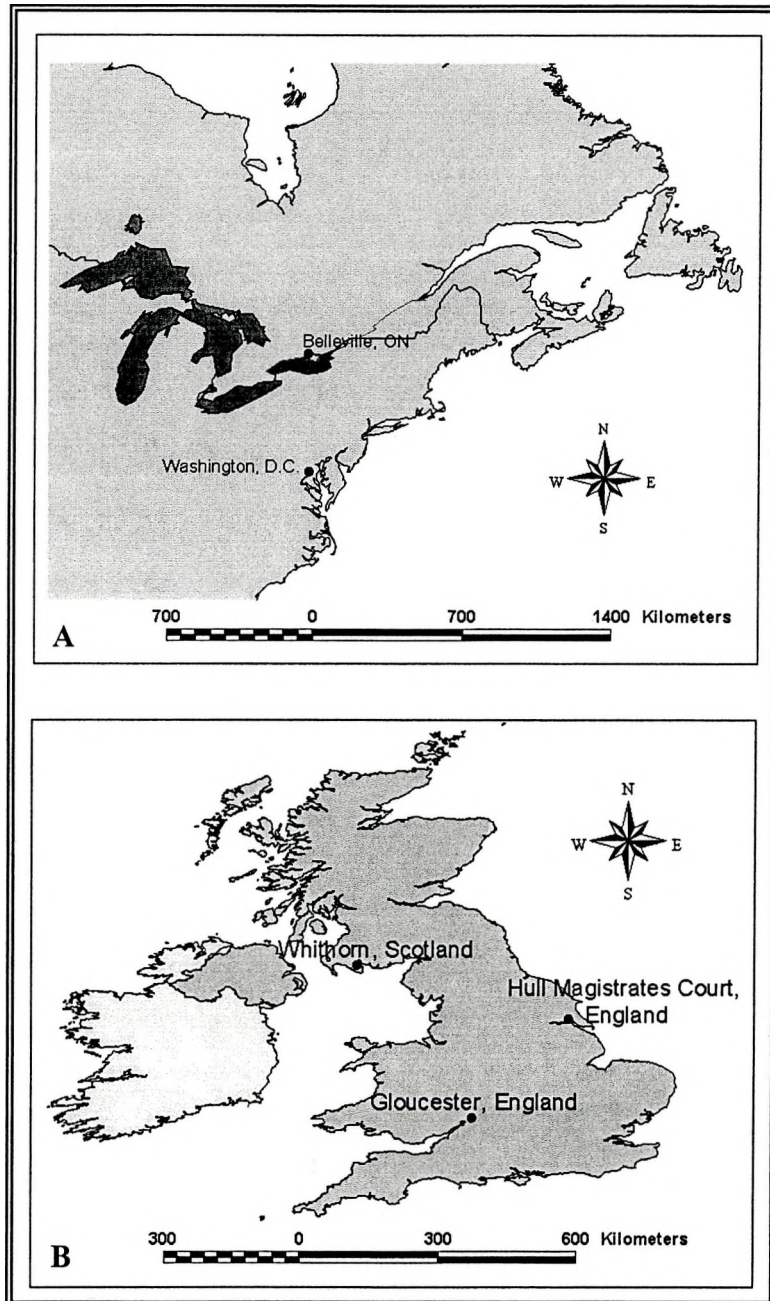


Figure 4.1: Maps illustrating geographical placement of individuals sampled for this project. It must be noted that the original living locations for all Civil War Period samples is unknown and only their site of curation (Washington, D.C., U.S.A.) can be used for placement.

	Origin	Sample #	Bone Element	Non/Pathological
1	<i>Civil War Period, USA</i>	1001949 MM2880-2	articular surface of tibia	np
			bone dust (no decontamination)	?
2		1001949 MM2880-4	articular surface of tibia	np
			bone dust (no decontamination)	?
3		1002700 MM1015 ^{mr}	fibula end	np
4		1001245	rib	np
5		1002814 MM1154 ^{mr}	skull?	p?
			skull	p (healed?)
			skull	p?
6		1000304 MM1622 ^{hd}	long bone	np?
			long bone with lots of spongy	np?
			bone dust with foil particles	?
7		1002422 MM530 ^{mr}	long bone (femur?)	p
	femur in foil		p	
	bone dust (no decontamination)		?	
8	295209	skull (shellac)	np	
9	1000873 MM2510	skull (black colouring)	np?	
10	<i>Hull, England</i>	HMC94-SK805	fibula	p
11		HMC94-SK932	humerus	p
			tooth	np
			long bone	p
12	HMC94-SK1121	fibula	p	
		right metatarsal	np	
		tibia	p	
13	HMC94-SK1216	scapula	np	
		long bone	p	
		tibia	p	
14	<i>Whithorn, Scotland</i>		healed? gumma	p
15	<i>Blackfriars, England</i>	SK77	rib	p
16	<i>Belleville, Canada</i>	B302	proximal radius	np
			mid femur	p

Table 4.2: Summary list of samples.

NP = non pathological 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

4.1.1.1 Civil War Time Period Samples:

Samples from nine of the sixteen individuals were acquired from the Armed Forces Institute of Pathology from Paul Sledzik in 1994 by D. Yang and S.R. Saunders.

All specimens were taken from individuals who died in the mid to late 1800s in the United States. These particular samples are most important because they have associated historical documentation indicating the presence of syphilis. Medical records obtained during treatment and/or autopsy provide support for the statement that these nine individuals suffered from syphilis. A summary of the transcribed information from the original documents is provided below.

MM 2880-2 and 1001949; MM 2880-4 and 1001949: These two entries include four tibiae that were described as being “enlarged and thickened by periostitis”. These bones were purchased from the Gibson Cabinet Collection in 1868 (Army Medical Museum, 1868). Due to the fact that these bones had been purchased and direct clinical/medical documentation does not exist, a tentative diagnosis of syphilis has been given.

1002700 (MM 10067: MS 4255): The one bone sample obtained from this individual belonged to a man from Co. L. of the 7th Cavalry. Bone was collected on August 7, 1890 during the autopsy by Major W.H. Forwood, Surgeon, USA, Attending Surgeon, conducted at the U.S. Soldiers’ Home, Barnes Hospital, Washington, D.C. The records (hand-written and typed) revealed that the right tibia and fibula showed “much irregular and general thickening of compact tissue and abundance of exostotic growths” (See Plate 4.1) (Forwood, 1890).



Plate 4.1: Right tibia and fibula of Civil War individual 1002700. Note presence of proliferative bone (periostitis and osteitis), especially in the distal end of the tibia. Photo courtesy of Paul Sledzik, AFIP

During the autopsy, when the skull was observed, no external marks were noticeable on the scalp, but the frontal and parietal bones were noted as being “extensively eroded in a manner similar to that of the legs, and the underlying dura was closely adherent to the brain, the latter was dark and congested over this area, but no gummata, tumor or abscess was found in it.”

Medical records also exist pertaining to examinations performed when the individual was alive. The records state:

[He] was admitted to Hospital July 14th, 1890 with what he claimed to be chronic rheumatism, but which proved on further examination to be an advance stage of tertiary syphilis. The early history of this case was difficult to obtain as he would not admit its specific origin; this would of course complicate any claim he might want to make for pension. He had been sick a good deal for several years and treated in various hospitals taking principally mercury and potassium iodide; of late he had acquired the morphine habit. Three years ago he was stricken with paralysis on the right side, the effects of which still remain in a right partial spastic hemiplegia. The muscles of that side are weakened and there are contractures of the leg and arm, the tendency being to flexion in the latter (arm) and extension in the former (leg). The head is drawn to the right and the right angle of the mouth drawn down; articulation is slow and difficult. The left leg is somewhat involved in the same manner but not so much as the right. Both feet and legs to the knees were enormously swollen with oedema. Obstinate diarrhea began soon after admission; his paralytic condition became general and deepened into coma and death ensued August 6, 1890.

1001245: AMM 5465: Specimens from this case were of an individual who had “antero-posterior curvature of the spine with caries of the bodies of the vertebrae” (See Plate 4.2). Bones from this case were purchased from the Gibson Cabinet (Otis, 1868a). As direct documentation of this individual is lacking, only a tentative diagnosis of syphilis can be made. The possibility exists that this individual had tuberculosis instead of syphilis. This possibility is based on direct observation of the visual documentation. The picture shows several vertebrae with lytic/erosive lesions causing slight kyphosis of the column and ankylosis of ribs to the vertebrae.

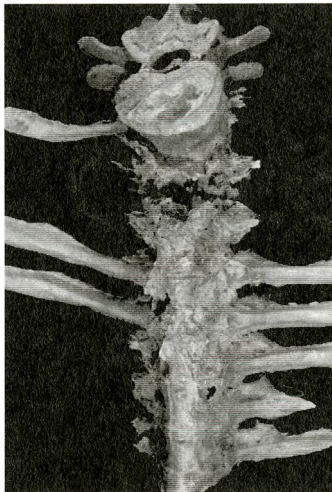


Plate 4.2: Thoracic vertebral column of Civil War individual 1001245. Note deformed angulation caused by erosion of several vertebrae. (Vertebral column is upside down for ease of picture taking.) Photo courtesy of Paul Sledzik, AFIP

MM 1154: 1002814: 10735 P.S.: Three skull pieces were obtained from this male aged 47 years. Records state that he died “of tertiary syphilis at Fort Mason, California, May 1894” Autopsy notes were provided by Wm. L. Kneedler, Captain and Assistant Surgeon, USA, Post Surgeon at the AMM June 13, 1894 (Kneedler, 1894). His records state that the “Dura mater showed thickened patches adherent on internal surface to the

bone. Calvaria showing thickening and exostoses, ulceration and perforation.” Plates 4.3a and b provide evidence of the irregular topography characteristic of caries sicca. The kidney was also identified as being ‘syphilitic’ Observations of the actual bone itself provided evidence of nodes, cavitations and stellate scarring which are characteristic of syphilitic infection.

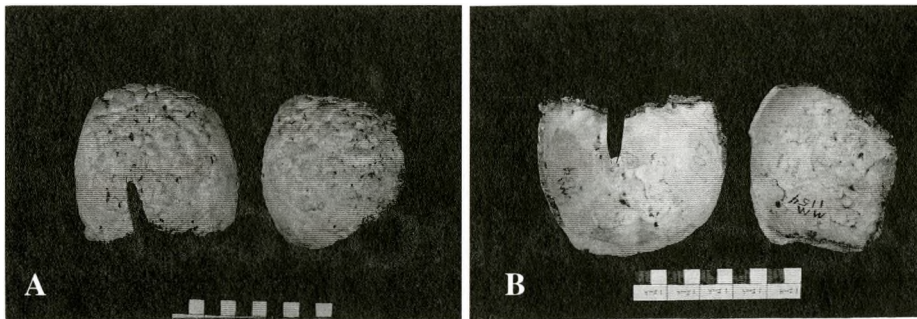


Plate 4.3: Civil War individual MM 1154. Ectocranial (A) view showing irregular topography caused by (healing) caries sicca. Endocranial (B) view also affected. Photo courtesy of Shelley R. Saunders.

MM1622: 1000304: AMM/P.S. 6251 & 6252: Diagnosis of syphilis in this individual was based on the descriptions written by Dr F.N Otis in 1883 (Otis, 1883). For example, Dr Otis states that the

Frontal bone shows bony loss perfectly characteristic of syphilis. Also, eburnated depressions in this bone near coronal suture in median line both ulcerative and non ulcerative. Bony loss of palatal bones left especially Inferior turbinated bones also absent. Also, left lacrimal. Also loss in roof of left orbit, just under superciliary ridge and one cicatricial (scar) depression in the glabella adjoining it. The syphilitic origin of the disease causing this destruction appears to me unquestionable.

This “syphilitic” cranium, right and left femora and tibiae were presented to the Army Medical Museum by Surgeon J W Brewer, U.S. Army on April 10, 1873. Found in the same donated box was a skull of a black bear and a badger as well. The human remains

are said to be that of an unknown individual, possibly Native American who died at 'Camp Supply, Indian Territory', 1873 (Otis, 1883). Due to the fact that this specimen was a donation and no medical records exist, a tentative diagnosis of syphilis is being used.

Observations of the skeletonized left femur (Plates 4.4a and b) reveal cloacae, lytic lesions, and much remodeled bone, especially large amounts of the latter two forms on the distal half of the femur. A large amount of remodeled bone and large lytic lesions are found on the proximal half of the left tibia (Plates 4.4a and b). Osteomyelitic cloacae are present in the proximal portion of the right tibia. These cloacae are possibly remnants of gummatous lesions.

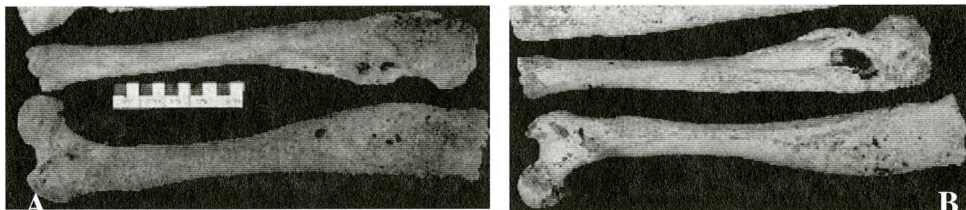


Plate 4.4: Civil War individual MM1622. Frontal (A) and distal (B) views of the right femur and medial (A) and lateral (B) views of the right tibiae. Note: osteitis, periostitis and several cloacae of the distal femur and proximal tibia. Photo courtesy of Shelley R. Saunders.

MM 530: 1002422: Dr D.S Lamb, Washington, D.C. performed the autopsy on this individual 10 hours after her death (Lamb, nd). His autopsy records state she was greatly emaciated and had an ecchymosed patch over the left eye (suggested it was from a fall). Over each knee she had an “abrasion with dryness and induration of skin, thick scabs a

foot in extent along the tibial crests and an inch or more in breadth”. Dr. Lamb goes on to write

The heart present some fatty degeneration of the anterior segment of the mitral valve, was otherwise normal. The ascending aorta showed a small patch of fatty degeneration of the lining membrane. There were pleuritic adhesions at the base of the right lung and between the lobe and oedema and hypostatic congestion of the lower lobe and some tubercles. There were firm red adhesions of the surface and between the lobes of the left lung; lung itself anaemic with tubercles. An intercalary cavity and bronchial inflammation in the upper lobe; lower lobe free from tubercles.

Dr. Lamb also observed “firm adhesions of the liver to the diaphragm” and amyloid degeneration of several organs (e.g., heart, spleen, kidney), as well as a number of blood clots in the stomach and intestines. Her “knee joints contained fluid and presented erosions of the cartilages and exostoses of the bones. The periosteum along the tibial crests was thickened by chronic inflammation and the adjacent bone presents a number of shallow erosions. There were also many exostoses of the bones of the legs and the adjacent muscles were infiltrated and fatty”.

Other records found in this specimen’s file state the bone shows ‘syphilitic inflammation’ with ‘osteomyelitis’, ‘osteoporosis’ and ‘ossifying periostitis’. The records go on to say that the “lower ends of femora with patellae, tibiae and fibulae, showing results of inflammation, probably syphilitic; surface of bone, especially of front of tibiae, very irregular, with marked depressions and new growths of bone; general osteoporosis” (Plate 4.5).

The autopsy report suggests tuberculosis to me, but the rest of the records specifically use the words “syphilitic” to describe the lesions and so, a diagnosis of syphilis has been tentatively made.



Plate 4.5: Left tibia and fibula of Civil War individual MM530. Increased thickness of bone caused by periostitis and osteitis and the presence of cloacae at midshaft and distal end. Photo courtesy of Paul Sledzik, AFIP

295209: P.S. 10032 & 10033: This specimen was purchased by the Army Medical Museum from the collection of the late Prof. F.H. Hamilton, New York, NY, 1886 (Army Medical Museum, 1886). Evidence of pathology is observed from the skeletal material and not from medical records. Observations of the cranium (Plates 4.6a and b) present areas with varying degrees of absorption and destruction of inner and outer tables amounting to complete absence in some places where large fenestra are seen. Evidence of hypertrophy and new growth are also noted. Erosion of the palate is also observed (Plate 4.6b). Information also found within this file state that her left tibia and fibula had a “simple fracture” around the middle third of the bones which occurred while “walking across the floor” Records indicate that these remains are said to be of a “funny old lady

who was an upper domestic” living in a hotel in Sandusky, Ohio, in 1842 as mentioned by Dickens in his “Notes of American Travel, Chapter XIV” She was over the age of 60 when she died of syphilis in 1868 or 1869 in the Almshouse at Norwalk, Ohio, under the care of Dr A.N Read.

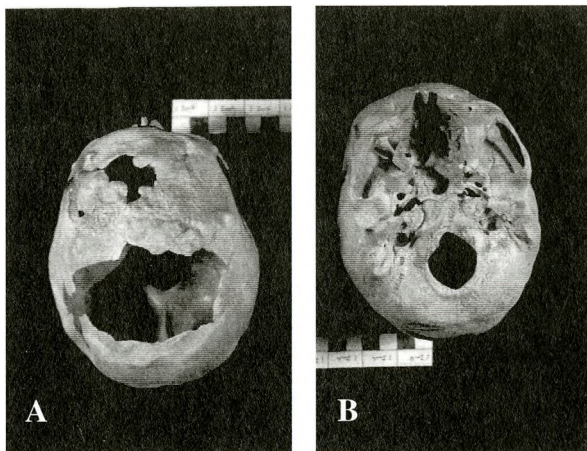


Plate 4.6: Civil War individual 295209 Superior (A) and inferior (B) views of skull. Erosion of inner and outer tables caused by a severe case of caries sicca. Also note the erosion of the palate. Photo courtesy of Shelley R. Saunders.

Located within this file were notes postulating the identification of this individual.

The notes read.

It is possible that the somewhat eccentric behavior of this serving-woman, as narrated by Dickens, might plausibly be accounted for by the fact that her brain was already attacked by the disease (syphilis) which was later to cause death. Her childish wonder and serious, unashamed contemplation of the great novelist would indicate some mental disturbance to explain such behavior in a woman approximately 40 years of age.

MM 2510: 1000873: 5105: This sample was purchased from the Gibson Cabinet by George A. Otis, Asst. Surg., USA, May 1, 1868. The only records obtained for this specimen state that the calvarium shows areas of ulceration of both parietals and the left temporal bone (Plates 4.7a and b) (Otis, 1868b). Since this sample was “purchased” and no medical records were found, only a tentative diagnosis of syphilis can be made.

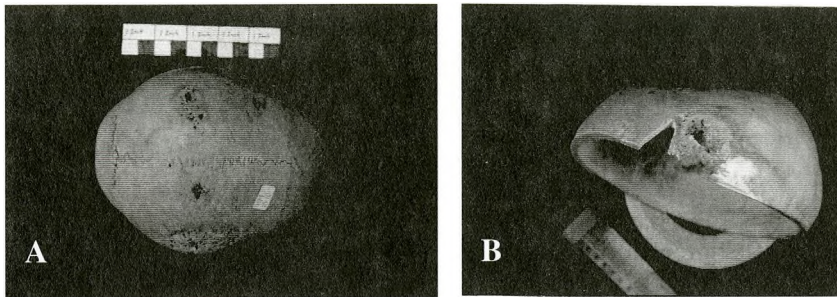


Plate 4.7: Civil War individual MM2510. Superior view of skull (A) with multiple areas of erosion in the parietals (possible caries sicca). Left side view of the calvarium (B) with a lytic lesion and area showing where the bone sample was taken. Photo courtesy of Shelley R. Saunders.

4.1.1.2 United Kingdom Samples.

Samples from six individuals were acquired from the University of Bradford skeletal collections from Charlotte Roberts and Anthea Boylston. These samples, with the exception of two (Whithorn gumma and Blackfriars SK77), were obtained by the author in the summer of 2000. The Whithorn and Blackfriars samples were sent via mail soon after the other United Kingdom samples were obtained. The Whithorn sample comes from a young adult female cranium described as having typical caries sicca (see Plate 4.8). This cranium was found amongst other disarticulated material from Period V (dated AD 1300-1450) of the late medieval cemetery component from the site of a monastic town in southern Scotland (Cardy et al., 1997).

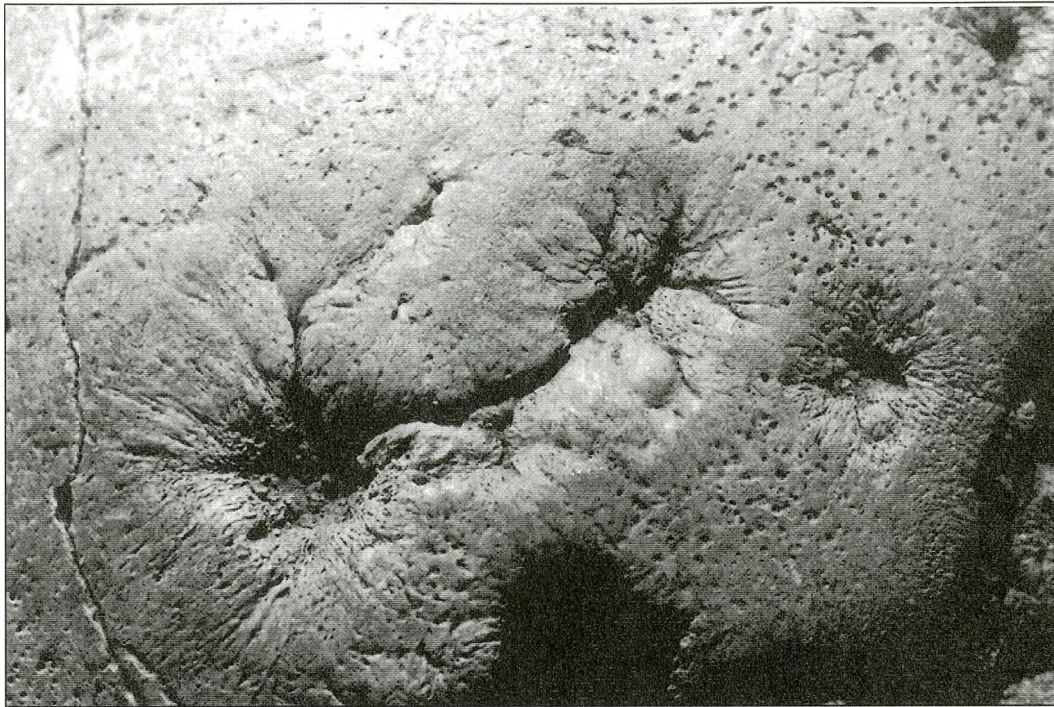


Plate 4.8: Whithorn calvarium showing several healed lesions indicative of caries sicca. Photo taken from Cardy et al. (1997:544) by permission from the publisher

Blackfriars SK 77 comes from a site in Gloucester, England. The site is a Dominican Friary of Blackfriars founded in AD 1239 with burial use documented around AD 1246 with dissolution of the friary occurring in AD 1538 (Atkin, 1993). The stratigraphic position of burial SK 77 suggests a contextual date of the mid-15th century (Atkin cited by Roberts, 1994). Complete information (site context, descriptions of pathology and pictures) on SK77 can be found in Roberts (1994) and Ortner (2003:310-11), but a quick synopsis is provided here.

Blackfriars SK77 was a young adult female individual who is described as having an advanced case of syphilis. Both proliferative and destructive bone lesions are noted. Healed stellate scars of caries sicca are identified on the occipital, right parietal and

frontal bones (Roberts, 1994:104). Destruction of the nasal aperture has occurred with perforation of the palate. Roberts (1994:104) and Ortner (2003:310-311) note that osteoproliferative lesions were found on the ribs, clavicles, scapulae, sternum, humeri, right forearm, right ilium, femora, tibiae and fibulae.

The remaining United Kingdom samples (Hull Magistrates Court - HMC) come from burials found at a medieval Augustinian Friary located in the now modern city of Kingston upon Hull excavated in 1994 (Evans, 2000). A major port of entry in northern England, Hull was a highly populated city with many transients. The site has been dated between AD 1300-1450 via dendrochronology of the coffins and about AD 1400 by ^{14}C (David Evans, personal communication). The stratification of the site suggests that the four specimens used for this research were buried around AD 1450-75. The aging and sexing information for these individuals from Hull was obtained from the original records documented by the osteologists working on the site (Anthea Boylston, personal communication) (Krogman, 1962; Loth and Iscan, 1989). A quick assessment was performed by the author with similar results.

HMC 94-SK805: This is an edentulous male (approximately 40-61 years of age) with eburnation on most joint surfaces. The third to the eleventh thoracic vertebrae are ankylosed (Plate 4.9). All of the upper limbs (humeri, radii, ulnae and clavicles) contain osteitis and/or periostitis to varying degrees. Osteoarthritis is present in the cervical and upper thoracic spine. All of the lower limbs (femora, tibiae and fibulae) (Plate 4.10) also show osteitis and/or periostitis with the right fibula (Plate 4.11) engulfed with irregular

topography produced by the presence of these two types of pathology. Even several metatarsals have spongy periostitis and osteitis.

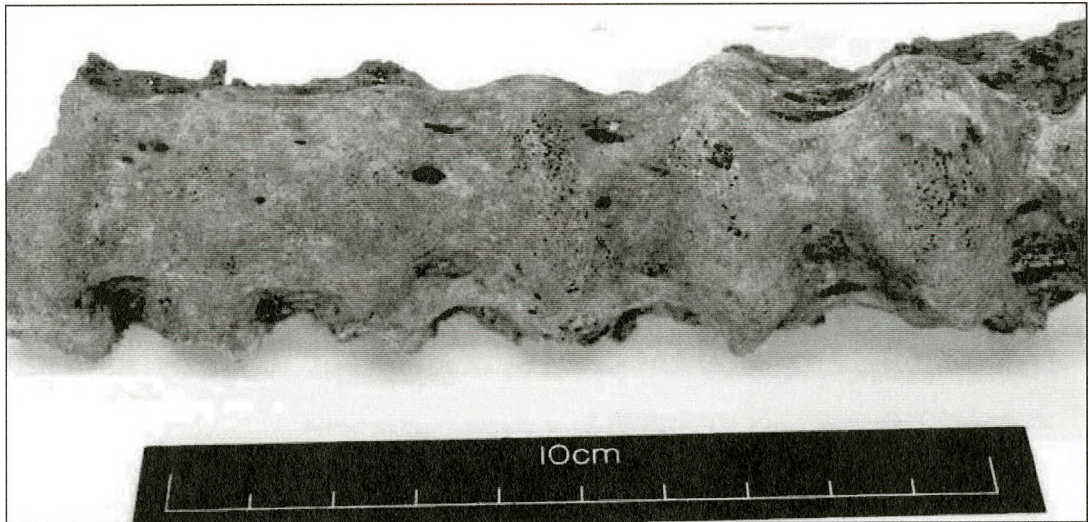


Plate 4.9: Individual HMC94-SK805. Frontal view of ankylosed thoracic vertebrae, most likely diffuse idiopathic skeletal hyperostosis (DISH).

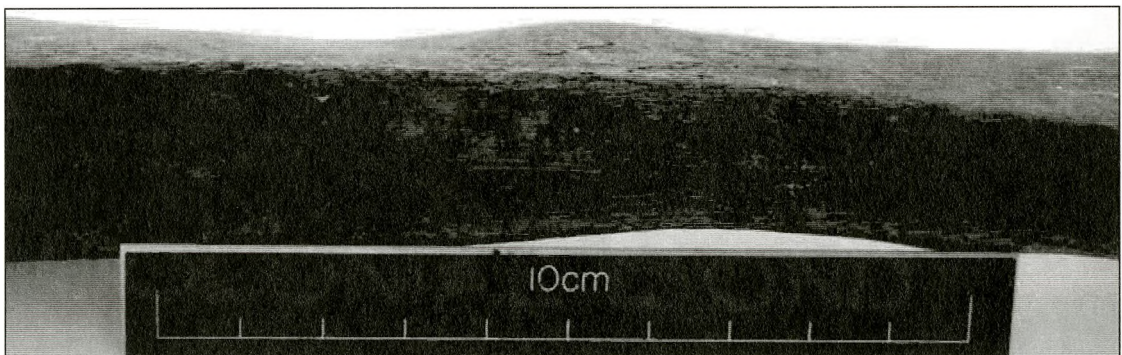


Plate 4.10: Individual HMC94-SK805. Left tibia with osteitis and periostitis (“bump” in the middle of the picture).

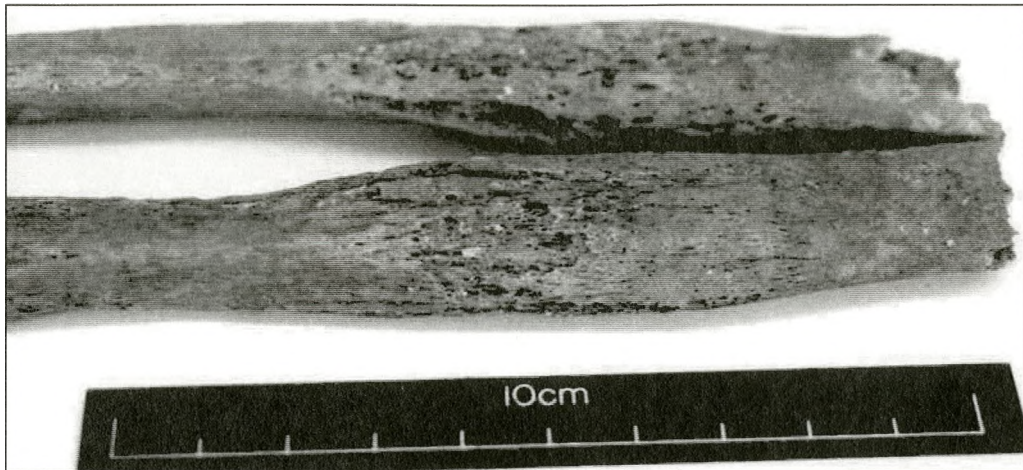


Plate 4.11: Individual HMC94-SK805. Right and left fibulae with osteitis and periostitis.

HMC 94-SK932: This individual is a male aged approximately 36-45 years. The remains are fragmentary with no complete long bones. The observed frontal bone pieces have what could be considered healed remnants of caries sicca. the outer table of bone is non-existent and the inner spongy bone is very dense with some areas of the calvarium quite thickened. Carious lesions are present on several teeth and the right first premolar is turned perpendicular to the norm. The dorsal distal side of the left humerus (Plates 4.12a and b) has a large area of ‘ballooned’ osteitis with some periostitis while the frontal side contains spongy periostitis. Small areas of osteitis are also observed on the shafts of the left radius and ulna. The left femur and both tibiae present with severe osteitis (‘ballooned’) with striated and spongy periostitis present only on the tibiae. Several areas of osteitis were also noted on the right fibula.

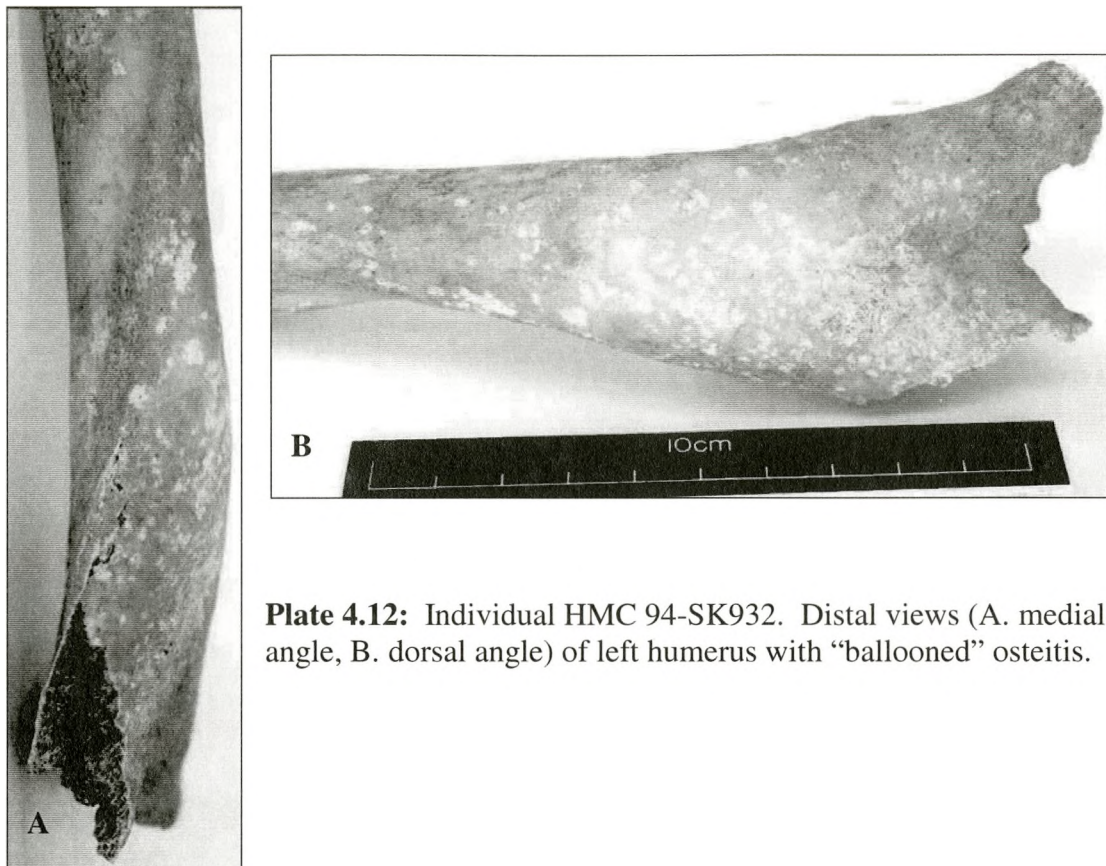


Plate 4.12: Individual HMC 94-SK932. Distal views (A. medial angle, B. dorsal angle) of left humerus with “ballooned” osteitis.

HMC 94-SK1121: This individual is a 25-39 year old female with slight cribra orbitalia in the left orbit. All of the upper limbs, including the scapulae and right clavicle, contain areas of osteitis and/or periostitis. All of the lower limbs show signs of severely marked osteitis (irregular topography) and striated and spongy forms of periostitis (Plates 4.13, 4.14 and 4.15).

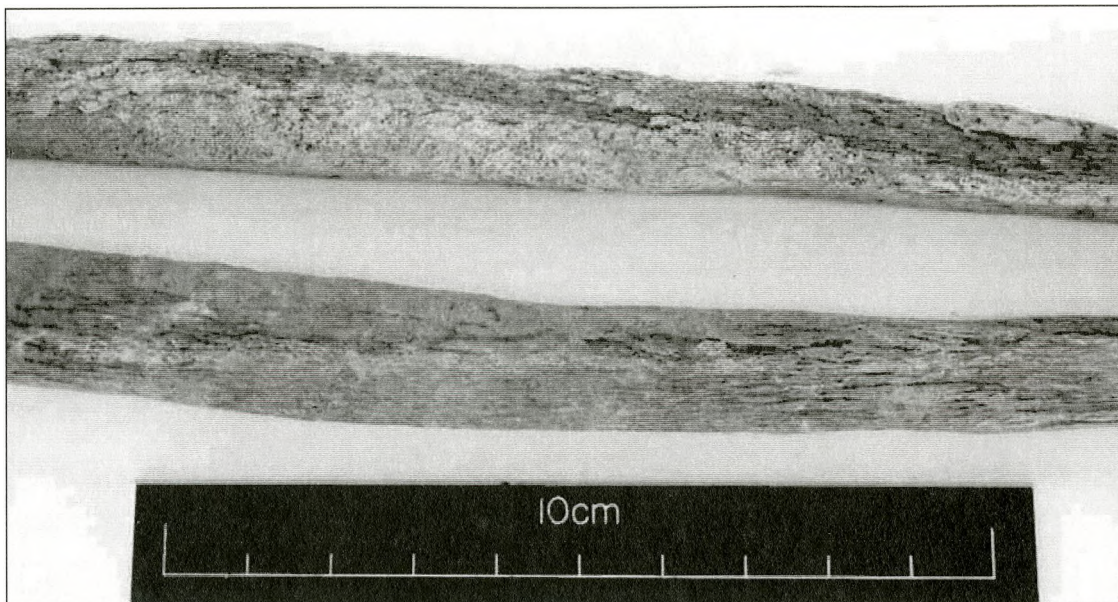


Plate 4.13: Individual HMC 94-SK1121 Left (top) and right (bottom) fibulae with osteitis and periostitis (striated and spongy).



Plate 4.14: Individual HMC 94-SK1121 Left (top) and right (bottom) tibiae with osteitis and periostitis (striated and spongy).

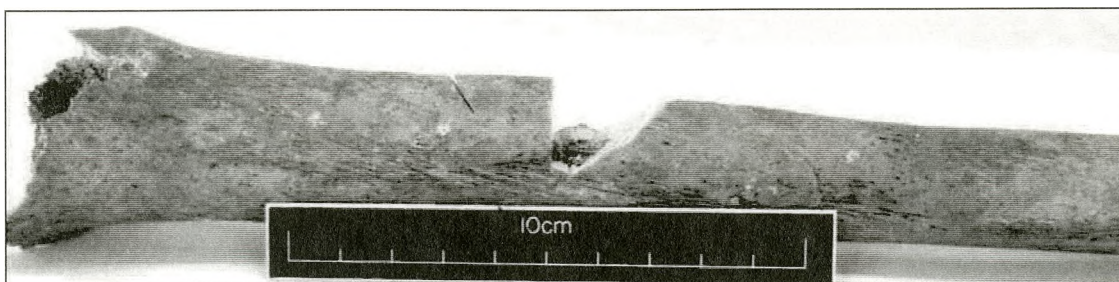


Plate 4.15: Individual HMC 94-SK1121 Right pathological tibiae showing sampled area.

HMC 94-SK1216: This individual is a young adult male approximately 20-35 years of age. No complete long bones exist for this individual. The skull presents with classic caries sicca on the frontal bone (Plate 4.16).

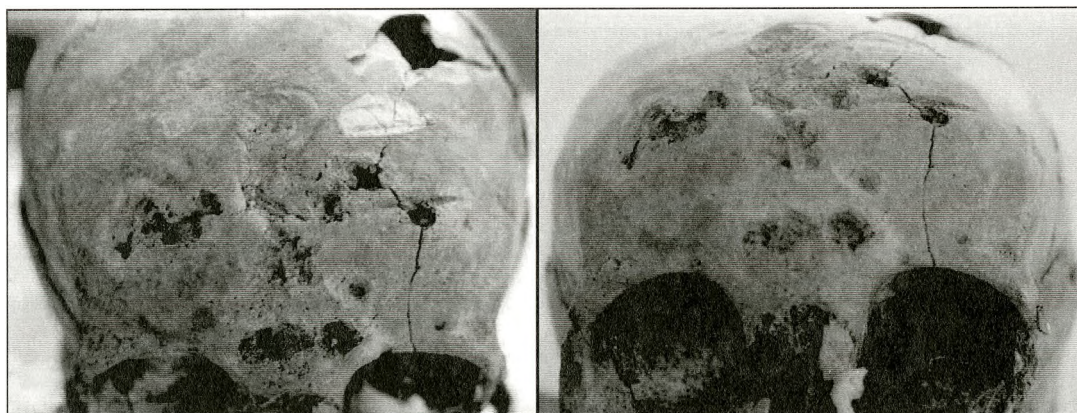


Plate 4.16: Individual HMC 94-SK1216. Frontal views of skull with erosive and healing lesions of caries sicca on the frontal bone.

Most of the lesion is erosive with several small areas beginning to heal. The left side of the nasal cavity contains spongy periostitis on the outer and inner rims. A symmetrical lesion of spongy periostitis and possibly osteitis can be found on the lower border of the mandibular body just inferior of the molars. An erosive cloaca/sequestra can be found on the acromion process of the right scapula. The right clavicle has severe periostitis at the midshaft region. The right and left humeri have areas of osteitis and periostitis at the distal dorsal end of the shaft. The right and left radii, as well as the left ulna, have severe osteitis and periostitis (spongy and striated) over most of the shaft with several cloacae/sequestra (exception: the right radius has only two erosive holes which do not penetrate to the medullary cavity). These cloacae may be evidence of gummatous lesions often found in syphilis. The shafts of the femora, tibiae and left fibula (Plate 4.17a) all show very severe osteitis and periostitis (striated and spongy) with the presence of 'snail tracks' (Plate 4.17b). This excess osseous material has made these bones quite heavy.

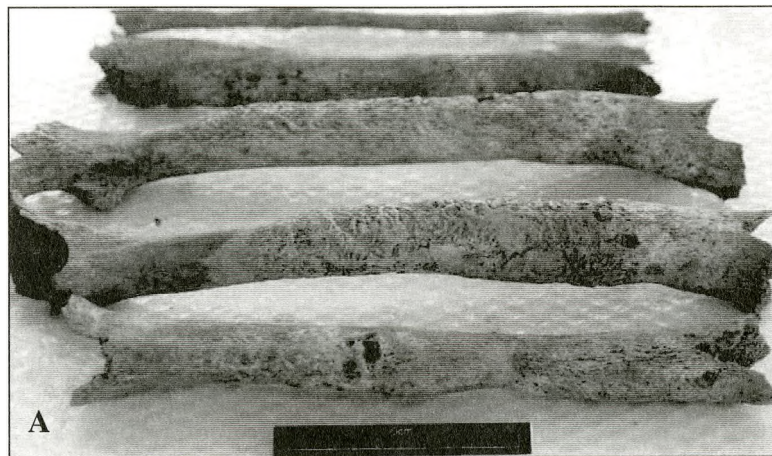


Plate 4.17: Individual HMC 94-SK1216. Lower limb bones (A) showing severe forms of pathology: osteitis, periostitis (spongy and striated) and cloacae from possible gummatous lesions. Close up of the femora (B) showing irregular topography with ‘snail tracks’ and numerous cloacae.

4.1.1.3 Ontario Sample.

Two bone samples (proximal radius and mid femur) from one individual were taken from the St. Thomas’ Anglican Church cemetery, Belleville, Ontario. This site has been dated to AD 1821-74. These samples belong to burial B302 who is a male approximately 60+ years. This individual has classic caries sicca on the frontal bone

(Plate 4.18) and osteitis and/or periostitis as well as numerous cloacae of the humeri (Plates 4.19a and b), clavicle (Plate 4.20) and femora (Plate 4.21) (Jimenez, 1991).



Plate 4.18: Individual B302 from Belleville. Caries sicca. Photo courtesy of Shelley R. Saunders.

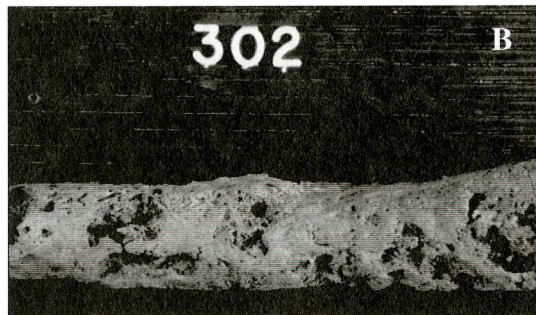
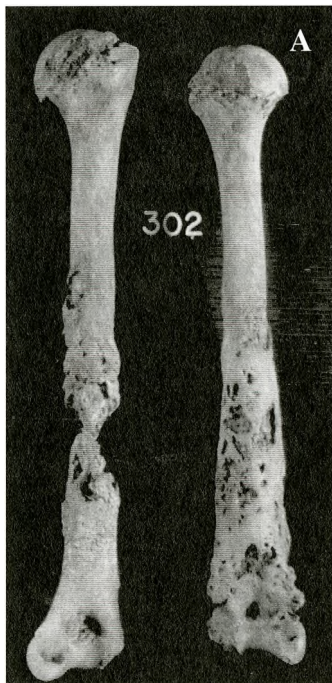


Plate 4.19: Individual B302 from Belleville. Osteitis and periostitis of left and right humeri (A) with massive erosion of lower midshaft of right humerus. Close up of left humerus showing multiple cloacae (B). Photos courtesy of Shelley R. Saunders.

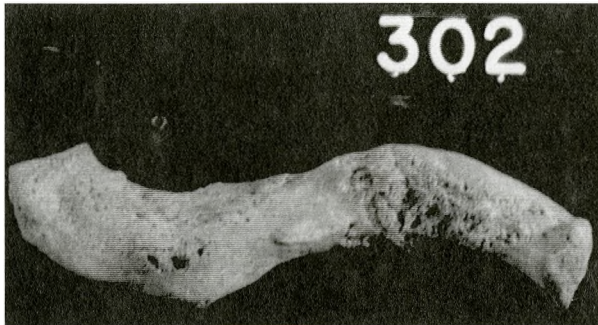


Plate 4.20: Individual B302 from Belleville. Left clavicle illustrating proliferation of bone (periostitis and osteitis). Note presence of several cloacae. Photo courtesy of Shelley R. Saunders.

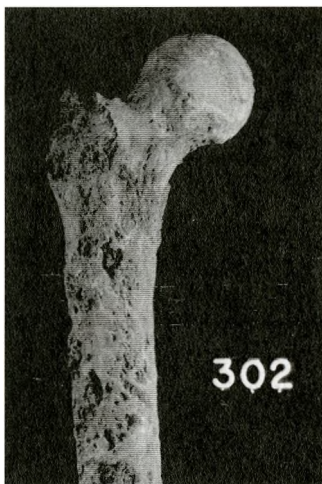


Plate 4.21: Individual B302 from Belleville. Left proximal femora illustrating periostitis, osteitis and cloacae. Photo courtesy of Shelley R. Saunders.

4.2 Histological Analyses

Histological thin sections were taken from several of the specimens and Schultz's (1994; 2001, 2003) criteria were applied. Those cases sampled include the Civil War MM530 femur, HMC94-SK805 fibula, SK932 proximal right humerus and long bone fragment, SK1121 fibula and tibia, HMC94-SK1216 tibial fragments, Blackfriars SK77 rib and Belleville B302 femur because they contained sufficient amounts of tissue which allowed for both aDNA and microscopic analyses.

Thin-sectioning of all bone samples were carried out in the McMaster University histology lab (with assistance by Patrick Beauchesne) following the protocol first set out by Pollet (1994). A brief explanation of the procedures is summarized here.

Approximately 1.5cm sections of cortical long bone were cut from the original samples and cleaned with a solution of 90% distilled water and 10% Sandison's Fluid (95% ethyl alcohol, 1% aqueous formalin, 5% aqueous sodium bicarbonate) followed by 100% distilled water in an ultrasonicator. Next, the samples were dehydrated in order to allow for proper embedding. Specimens were washed in three grades of ethyl alcohol solutions (50%, 75% and then 100%) in an ultrasonicator. After the last alcohol wash, the samples were placed in a desiccator and allowed to dry over night. Next, they were embedded in a plastic resin (EPO-KWICK™ fast cure epoxy and hardener, Buehler) and placed in a vacuum apparatus in order to rid the embedded material of air bubbles. After 15-20 minutes the embedded bone was taken from the vacuum apparatus and allowed to harden for at least 24 hours. Once the embedded material was hardened, each sample was cut at a cross-sectional plane through the widest point of bone using an Isomet™ Low Speed Saw. The cut sample was then polished using an Ecomet III™ Polisher-Grinder (Buehler) and affixed to a glass slide by Thin Section Epoxy Part A and B™ (Hillquist). The slide was allowed to dry for at least 24 hours and another large cross-sectional cut was taken leaving a very small thickness of embedded bone affixed to the slide. This was then ground and polished to a desired thickness (e.g., 30-50 microns) using Carbimet™ Paper Disks of 320 grit to allow for proper analysis under a light microscope. When the final, desired thickness was achieved, a slip-cover was mounted onto the embedded specimen.

The prepared histological thin sections for this project were observed at 100X magnification under a light microscope (Olympus BH-2) and using polarizing filters.

Pictures were taken with a mounted Polaroid Digital Microscope Camera and imported into Adobe Photoshop 5.5 for analysis. A general assessment of the bone quality was first performed using a 'Histological Index' designed by Millard (2001). This index ranks histological bone sections from 0-5 with the bottom of the scale (i.e., 0) signifying no original features identifiable except possibly Haversian systems to the top of the scale (i.e., 5) where structures are very well preserved and virtually indistinguishable from modern bone. The presence of the 'Maltese cross' was also decided as a set criteria to observe as it denotes good preservation of lamellae and mineralization in osteon structures. After this was performed, the criteria set out by Schultz (1994; 2001) were used. If the histological thin sections lacked the presence of Grenzstreifen or Polsters with Achatknochen, then the structures were described and compared to other pathological conditions and alternative diagnoses were given.

4.3 Molecular Analyses

Three different DNA analyses were used for all samples in this project to understand or estimate the level of DNA preservation. The first measure of DNA preservation involved using mitochondrial DNA amplifications. Mitochondrial DNA is the most abundant endogenous DNA found in the human body with approximately 1000-10 000 copies per cell. As such, if it cannot be amplified from a sample during PCR, then the probability of being able to amplify nuclear or even pathogenic DNA is quite unlikely. For the second test of DNA preservation, sections of the amelogenin gene found on the X and Y chromosomes were amplified for each sample. This provided information regarding nuclear DNA preservation as well as the sex of the individual

sampled. Finally, treponemal DNA amplifications were attempted using the protocols described in Kolman et al.'s (1999) and Liu et al.'s (2001), a modification of Zoechling et al.'s (1997) and the newly manufactured *ARMS* assays on the samples. This approach provided insight into the differential preservation of DNA within the samples used for this project.

4.3.1 Rabbit Testing:

T.p. subspecies *pallidum* cannot be grown *in vitro* for extended periods of time (three weeks is the maximum so far [D. Cox, personal communication]) and as a result, the disease must be propagated and passaged through rabbits to provide viable organisms/DNA for testing purposes. In comparisons to human clinical symptoms rabbits do undergo similar primary and secondary presentations of the disease, but they do not exhibit signs of tertiary syphilis (Baughn and Musher, 1998). These experiments, along with the dissemination of the treponeme to a variety of organs and environments within the adult male New Zealand White rabbit (e.g., testes, skin injection sites, inguinal [groin] lymph node, spleen, heart, brain [Wicher et al., 1998], and blood [Wicher et al., 1992]), have already been tested via PCR except for the medium of bone.

Several different tissue samples were taken from two different types of adult rabbits donated by Dr. Hsi Liu from the Center for Disease Control and Prevention (CDC) in Atlanta: 'Rabbit #12' (samples taken from three of the 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. These two rabbits were in different stages of the disease with the former being in the later stage as all lesions where injections took place had healed by the time of sampling, and the second rabbit still having lesions at the site of injection with live spirochetes having been harvested that same day. All the aforementioned PCR protocols (see pages 89-90 for more details) were used to try to isolate treponemal DNA from these various tissue types.

4.3.2 Decontamination and Preparation of Skeletal Samples:

Each bone (approximately 1 gram sample) and whole tooth sample were subjected to a 1.5 minute soak in 1N HCl, then transferred to 1N NaOH solution for 1.5 minutes, followed by several rinses (approximately 10-20 minutes each) in (“ultrapure”) DNA/RNA free water. The samples were then placed in a UV cross-linker and rotated every 20 minutes until all sides were irradiated.

When the samples were dry, they were ground to a powder in a SPEX™ liquid nitrogen grinding mill.

4.3.3 DNA Extraction:

Extractions were performed on different days for those individuals where multiple samples were taken. Non pathological and pathological bone were also prepared on separate days. These two precautionary measures were used to help ensure no cross-

contamination could occur between samples from the same individuals and from different states of osseous response.

The powders of bone and teeth were mixed with a lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 100 μ g/ml proteinase K) in a sterile 15ml conical tube and placed in a rotating hybridization oven overnight (approximately 12-24 hours) at 55°C. The slurry was then centrifuged for 20 minutes and 2ml of the supernatant was placed in a Centricon (Fisher Scientific) for DNA concentration at 6500rpm for 30-90 minutes. When only 100-200 μ l were left, this volume was placed in a Qiagen QIAquick™ silica-based spin column for DNA purification. The final DNA solution (approximately 100 μ l) was used in subsequent PCR amplifications. Blank extractions containing lysis buffer only (no DNA) were carried throughout the experiment to assess the presence of exogenous DNA contamination.

Tissue extractions of the rabbit material were accomplished using the QIAamp DNA Mini Kit™ in a laboratory where no human, faunal or pathogen work had been performed before. 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.

4.3.4 PCR Conditions:

Amplifications were carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) in a 50 μ l reaction volume. The specific protocols are noted below for each area that was amplified. For all PCRs at least one negative control was

used (PCR master mix with no DNA added) and for mtDNA (K562 – Invitrogen) and treponemal DNA (see “clones” section) synthetic positive controls were used to assess contamination and PCR success, respectively. Each of the PCR amplifications was carried out with an initial denaturing of 94°C for 12 minutes and a final extension of 72°C for 7 minutes.

4.3.4.1 mtDNA: 50 mM KCl and 10 mM Tris-HCL, 2-3 mM MgCl₂, 200 μM dNTPs, 1.0 mg/ml BSA, 30 pmoles (0.3 μM) of each primer, 5 μl aDNA sample and 2.5 and 5 Units AmpliTaq Gold polymerase (Perkin-Elmer). When samples did not provide any results (presence of band on gel) on the first try, MgCl₂ and AmpliTaq Gold were increased to try to overcome the presence of possible inhibitors. Forty cycles of PCR at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. This reaction amplified a part of the HVRI region (16139-16420 nps) that is 281 basepairs (bps) in length.

Primers: **mt2-1** (5'ATACTTGACCACCTGTAGTAC) and
 mt2-2 (5'TGATTTACGGAGGATGGTG)

4.3.4.2 Amelogenin: 50 mM KCl and 10 mM Tris-HCL, 2.5 mM MgCl₂, 200 μM dNTPs, 1.0 mg/ml BSA, 50 pmoles (0.5 μM) primer Amel-Com, 15 pmoles (0.15 μM) primer Amel-Xa and 30 pmoles (0.3 μM) primer Amel-Ya, 5 μl aDNA sample and 2.5 and 5 Units AmpliTaq Gold polymerase (Perkin-Elmer). Fifty cycles of PCR consisted of 94°C for 40 s, 50°C for 30 s, and 72°C for 30 s.

Three primers (see below) were used to amplify the amelogenin gene on the X and Y chromosomes. This system uses a 154bp (479-633nps) and a 119bp (480-599nps) DNA segments for the X and Y chromosomes, respectively. Primers already exist for sex determination using the amelogenin gene (which result in 106 and 112 bp difference [Mannucci et al., 1993]), but this system often shows allelic dropout with the Y chromosome creating false identification of females. This occurs because the X chromosome is preferentially amplified due to its smaller length (106 bps). Also, the new system provides more separation between the two bands to allow for a better

identification of sex and to select for the Y chromosome oligonucleotide over the X chromosome. This ensures that sex can be identified even if the X allele drops out as a result of competition during the amplification cycles.

Primers: **Amel-Com** (5' TCATGAACCACTNCTCAGG),
Amel-Xa (5' CTGATTCTAAGATAGTCACA) and
Amel-Ya (5' GGTAATACTAACTTTGGGCA)

4.3.4.3 *Treponema pallidum* subsp. *pallidum*:

The Kolman et al. (1999) method was used on all tissue samples from the sixteen archaeological individuals and the two rabbits, but with slight modifications of the cycling parameters. Adjustments had to be made in order to accommodate for the use of different thermocycler machines. Denaturation, annealing and elongation times were shortened from 1 min, 2 mins and 1 min to 20 s, 30 s and 20 s, respectively. Beyond this alteration, the rest of the methodology was followed.

Two other genes, *polA* and 47-kDa membrane protein, were also isolated based on previously published methods (Liu et al., 2001 and Zoechling et al., 1997).

4.3.4.3.1 *polA*: A segment of this gene (377 bps) was isolated using Liu et al.'s (2001) protocol with a few exceptions: AmpliTaq Gold polymerase (Perkin-Elmer) was used instead of Expand high-fidelity *Taq* polymerase (Roche Molecular Biochemicals); GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) was used instead of model 9700 thermocycler (Applied Biosystems, Foster City, California); 1.0 mg/ml BSA was added.

4.3.4.3.2 47-kDa: Zoechling et al. (1997) provide a nested PCR system that was altered for this analysis. The original larger amplicon of 379 bps was not isolated first and nested with internal primer pairs, resulting in a 196 bp sequence isolation. Instead, only the 196bp amplicon was chosen due to its small length and possible better sequence preservation. PCR parameters were as follows: 50 mM KCl and 10 mM Tris-HCL, 1.6 mM MgCl₂, 200 μM dNTPs, 1.0 mg/ml BSA, 25 pmoles primers (see Zoechling et al.,

1997), 5 μ l aDNA sample and 5 Units AmpliTaq Gold polymerase (Perkin-Elmer). Forty cycles of PCR consisted of 94°C for 30 s, 67°C for 30 s, and 72°C for 30 s.

The 15-kDa lipoprotein and GPD genes were selected for the *ARMS* system because they have been sequenced and are useful in differentiating between the human venereal and nonvenereal as well as human pathogenic and nonpathogenic treponemes.

4.3.4.3.3 15-kDa lipoprotein gene: 50 mM KCl and 10 mM Tris-HCL, 1.75 mM MgCl₂, 200 μ M dNTPs, 30 pmoles (0.3 μ M) of each primer, 5 μ l aDNA sample and 2.5 Units AmpliTaq Gold polymerase (Perkin-Elmer). Fifty cycles of PCR at 94°C for 20 s, 69°C for 20 s, and 72°C for 20 s. This reaction creates a 147bp fragment (-171 to -25 nps).

Primers: **ARMTPALIPO** (5' GCGTGTTCTTGAGCGTCTGAGC) and **TPALCLIPO** (5' CAACTGGGTATACACCTACACAAG)

4.3.4.3.4 GPD gene: 50 mM KCl and 10 mM Tris-HCL, 1.75 mM MgCl₂, 200 μ M dNTPs, 30 pmoles (0.3 μ M) of each primer, 5 μ l aDNA sample and 2.5 Units AmpliTaq Gold polymerase (Perkin-Elmer). Fifty cycles of PCR at 94°C for 20 s, 67°C for 20 s, and 72°C for 20 s. This reaction creates a 169bp fragment (434-602nps).

Primers: **ARM579GPD** (5' ACATACACTAGATCCGATCCTCTT) and **ARM579GPD** (5' AGTTGCAGTTTATCCGTGGGTTG)

4.3.5 DNA Analysis and Sequencing:

PCR amplification reactions were run out on 8% non-denaturing polyacrylamide gels at 200V for 30 minutes. Positive samples were purified using Qiagen QIAquick™ PCR Purification Kits and sequenced in forward and reverse (not performed for multiple samples of same individual when forward sequences matched) directions using the primers mentioned above via an automatic sequencer, ABI PRISM® 3100 Genetic Analyzer, at a central facility on the McMaster campus (MOBIX). Sequences were then analyzed via BioEdit (Hall, 1999) against the Cambridge Reference Sequence (Anderson

et al., 1981) for polymorphisms in mtDNA and against *T.p.* subsp. *pallidum* (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 a computer program called mtRadius (Fluxus Technology Ltd.) in order to calculate haplogroup affiliation. The program mtRadius is a geographic information system which searches for the closest matches to a given mtDNA control region sequence and illustrates their geographic placement on a map (Röhl et al., 2001).

4.3.6 Cloning:

Treponemal DNA samples (*T.p.* subsp. *pallidum* Nichols Strain and Street Strain 14, *T.p.* subsp. *endemicum* Bosnia and *T.p.* subsp. *pertenue* Gauthier) were graciously donated by Dr. Hsi Liu of the Centers for Disease Control and Prevention (CDC), Atlanta. These DNA samples were used to start the project, but it was soon realized that there would not be enough DNA to sustain the number of tests needed. Consequently, it was thought that a renewable resource, which cloning offers, would be more appropriate. This would also ensure the presence of specific polymorphisms indicative of the venereal and nonvenereal pathogens that were needed for this project (Figures 4.2 and 4.3).

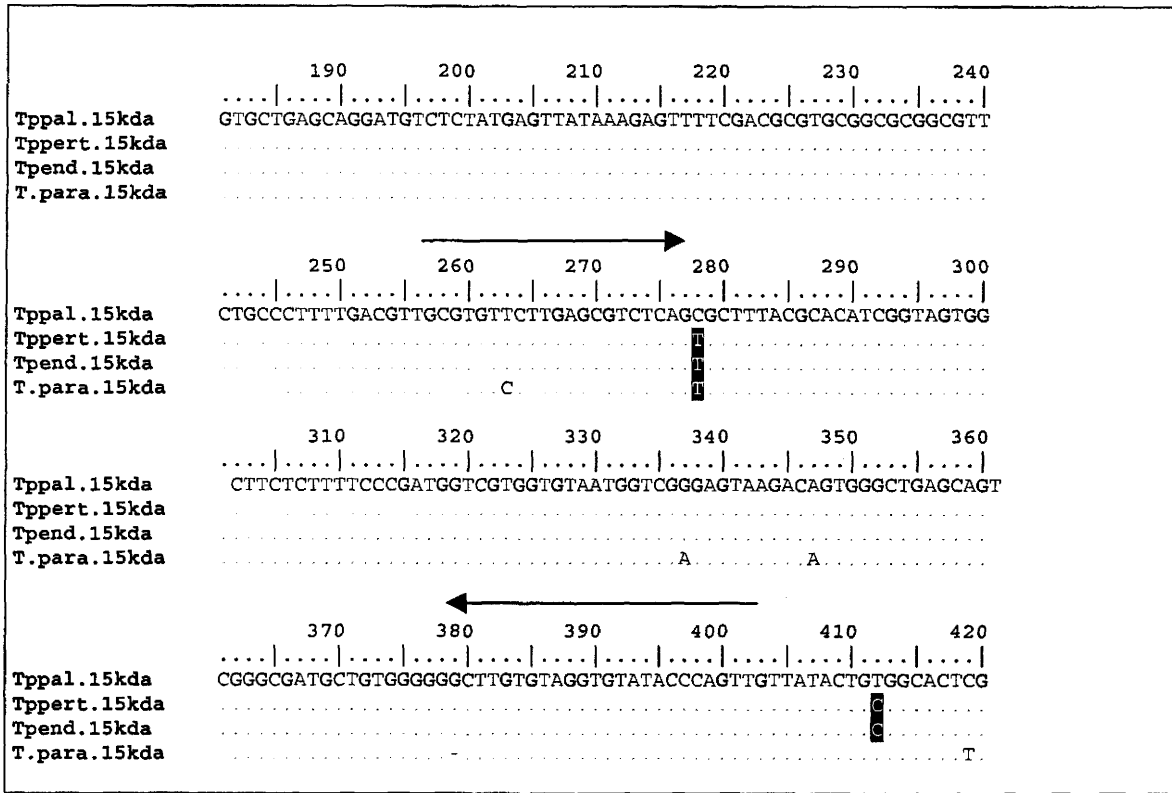


Figure 4.2: Alignment of 15-kDa lipoprotein showing sequence differences and amplicon target (147bps). Sequences taken from GenBank Accession Numbers U73115 (*Nichols-pallidum*), U73117 (*Gauthier-pertenuis*), U73118 (*Bosnia-endemicum*) and U73119 (*Cuniculi A-paraluiscuniculi*), respectively. The arrows represent primer placement.

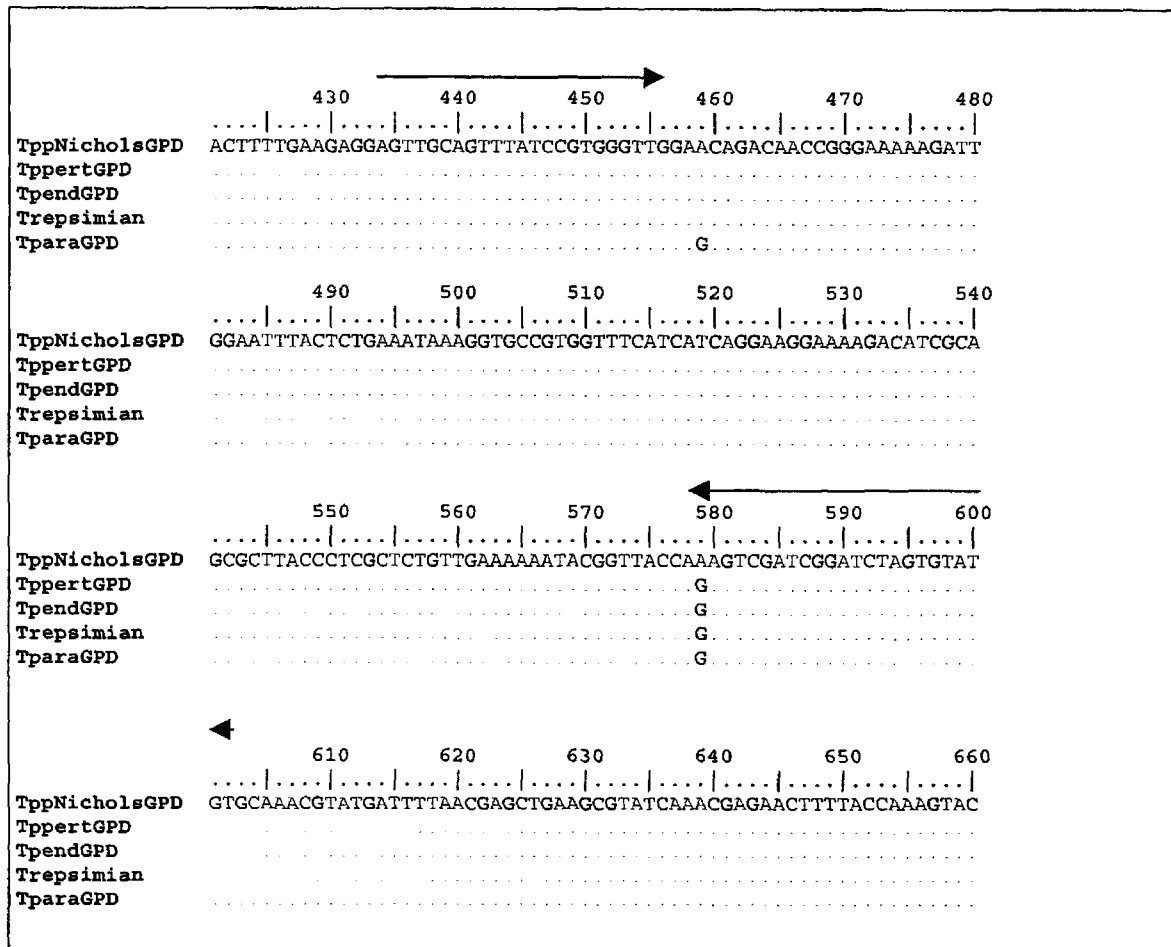


Figure 4.3: Alignment of GPD gene showing sequence differences and amplicon target (169bps). Sequences taken from GenBank Accession Numbers AF004286 (Nichols - *pallidum*), AF127422 (Gauthier - *pertenuis*), AF127423 (Iraq B - *endemicum*), AF127424 (Simian) and AF127425 (Cuniculi A - *paraluiscuniculi*), respectively. The arrows represent primer placement.

As such, clones were manufactured that possessed the correct nucleotide differences between the species as well as a section of deleted DNA so that the synthetic clones could be differentiated by amplicon size from 'true' treponemal DNA. This differentiation in

length is based on primer design, original primers would be used at one end of the sequence, but the other primer would have an addition. This addition incorporated a sequence complementary to a downstream section which when annealed to the DNA template, created a 'tail' (Figure 4.4). This meant that when run out on a non-denaturing 8% polyacrylamide gel, the synthetic clones would migrate faster due to their smaller amplicon size.

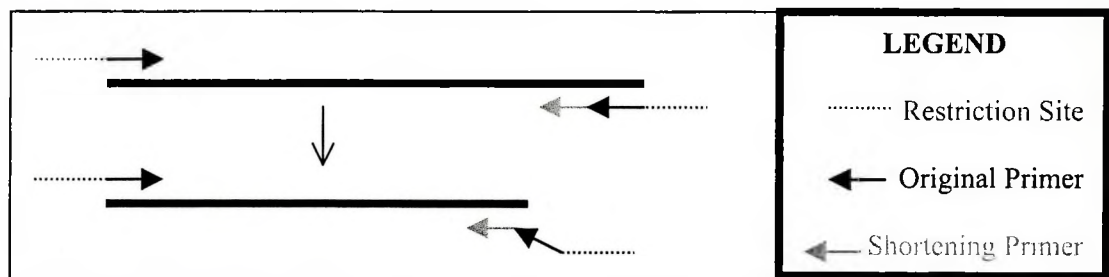


Figure 4.4: Diagram illustrating length differences due to primer design.

Two types of clones were synthesized for the two genes used in this analysis: a positive and a negative. The positive control was that which matched the *T.p.* subsp. *pallidum* strains and the negative control was that which matched non-venereal treponemes (e.g., *T.p.* subsp. *endemicum* Bosnia and *T.p.* subsp. *pertenue* Gauthier) for the chosen areas of analysis (refer to the alignments in Figures 4.1 and 4.2 for base pair differences).

The positive and negative controls were synthesized by designing primers (see below) that incorporated the distinct nucleotides differentiating these treponemal subspecies. These new oligonucleotides prime off of the CDC treponemal sample (i.e., *T.p.* subsp. *pallidum* Street Strain 14) and amplify during the polymerase chain reaction creating multiple copies of two distinct 'species' for the areas of the genes under study

Restriction sites (EcoRI [GAATTC] and BamHI [GGATCC]) were also incorporated into the primers to allow ligation into any plasmid with these two restrictions sites in their multiple cloning areas.

The six oligonucleotides used in this approach are:

Pallidum-specific 5' primer (15ARMSREP) for the 15-kDa lipoprotein gene:

GAGGAATTCGCGTGTTCTTGAGCGTCTCAGC*

*bp difference

Nonpallidum-specific 5' primer (15ARMSREE) for the 15-kDa lipoprotein gene:

GAGGAATTCGCGTGTTCTTGAGCGTCTCAGT*GCTTTAC

3' primer (15COMMON) common to both subspecies for the 15-kDa lipoprotein:

TCCGGATCCAACTGGGTATACACCTACACAAGCTGCTCAGCCCACTGTCTTAC

Pallidum-specific 3' primer (GPDARMSREP) for the GPD gene:

GAGGAATTCACATACACTAGATCCGATCGACTT*

Nonpallidum-specific 3' primer (GPDARMSREE) for the GPD gene:

GAGGAATTCACATACACTAGATCCGATCGACTC*TGG

5' primer (GPDCOMMON) common to both subspecies for the GPD gene:

TCCGGATCCAGTTGCAGTTTATCCGTGGGTTGTACTCTGAAATAAAGGTGCCGTG

The gray coloured bases represent the restriction sites (6bps) and the bolded section represents the primer that is actually situated upstream or downstream, depending on the primer, from the actual end of the DNA sequence which is complementary to the original *ARMS* primers used for this project (see PCR conditions above). This design renders the new cloned sequence shorter than the original (i.e., 15-kDa lipoprotein 127bp vs. 147bps

and GPD 139bps vs. 169bps) and helps to ensure that when the control is generated and run out on a gel, it will migrate at a faster rate. This type of control is important to help the researcher recognize both PCR success (presence of a band) and contamination (a specific migration rate or size of band) (Figure 4.5).

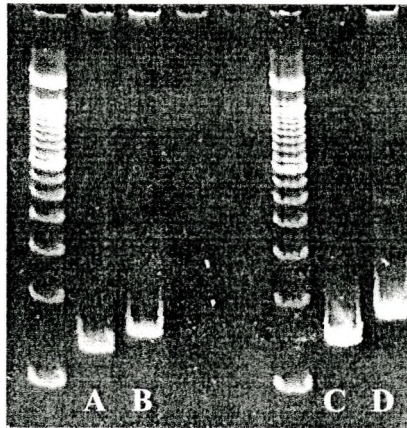


Figure 4.5: 8% polyacrylamide gel showing migration differences between the clone and the original DNA sample. A) 15-kDa lipoprotein *Pallidum* Clone, B) 15-kDa lipoprotein *Pallidum* Control, C) GPD *Pallidum* Clone, D) GPD *Pallidum* Control

Cloning was performed using the TA Cloning® Kit (Invitrogen™ Life Technologies) in a modern DNA laboratory separate from the MPI laboratories. This particular modern DNA lab has never analyzed syphilitic DNA prior to this project, thus negating the possibility of contamination. This kit provides all of the essential components which allow for the ligation of the PCR product into a vector/plasmid (pCR® 2.1) and transformation into competent cells. Chemically competent *E. coli* TOP10F cells were plated and grown overnight on Luria-Bertani (LB) agar with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) and IPTG (isopropyl- β -D-thiogalactoside) to provide blue-white screening of positive colonies. Positive colonies were picked and grown

overnight in LB broth/media and then the plasmid DNA was purified using the QIAprep® Miniprep Kit. The presence of an insert in a colony of plasmids was identified first by restriction enzyme digestion with EcoRI run out on both agarose and 8% non-denaturing polyacrylamide gels. When the insert was present (notified by the presence of two bands: one at the top representing the plasmid and one near the bottom representing the insert), it was then sequenced using the universal M13 Forward primer and in some cases using the original common ARMS primers (i.e., TPALCLIPO and ARM579GPDC). Only those clones that contained the correct sequences were used for further PCR reactions.

Positive (pallidum) and negative (nonpallidum) clones were used to set up the assays for the two genes. This required a magnesium titration, annealing temperature alterations and cycle number thresholds. The magnesium titration demonstrated that 1.5mM MgCl₂ works best for both genes. The annealing temperature for the 15-kDa lipoprotein assay works best at 69°C and at 67°C for the GPD assay. Finally, both systems can undergo 45 cycles without showing any problems (e.g., mispairing). Therefore these system parameters were used for the remainder of the amplification reactions.

4.4 Limitations of Methods

The author is aware of the fact that although the methodologies employed in this thesis project were used with the best intentions, limitations of their applicability do exist. For example, heavy reliance placed on autopsy reports performed during the Civil War time period may have created erroneous diagnoses as their accuracy is affected by the

relative medical understandings of that specific time period. Also for those medical notes which had been handwritten by a surgeon and for those which had been transferred to a typed document years later, transcription errors could have occurred. Observation of skeletal element(s) performed by me was an attempt to try to corroborate the diagnoses from the past, but in the end these are still “putative” diagnoses.

It is also recognized that bone can only react in so many ways which limits its appearance both at a macroscopic and microscopic level. The author is also aware of the fact that microscopic analysis of infectious disease, and in particular venereal syphilis, is limited and more work in the area is needed for a better understanding and characterization. Molecular analysis of treponemal organisms is also limited to the current published data and the author realizes that with more research (both personally and abroad) more sensitive assays can be developed and more genes (as well as entire genomes) will be sequenced providing better comparative data for a project such as this. Finally, it is recognized that the sample size used in this project is small which limits the probability of the identification of the bacterium, as well as not being epidemiologically applicable or representative of the population he/she once lived in.

CHAPTER FIVE:

RESULTS

5.1 Histological Analyses

Histological bone analyses were performed for seven of the 16 individuals (refer to Chapter 4) (see Appendix for macroscopic illustrations of histological samples). Of these seven, observations of more than one area of the skeleton were completed for two individuals (UK HMC94-SK932 and SK1121). This allowed for comparison of disease reactions and/or histological structure preservation within one individual. A summarized view of the results is presented in Table 5.1

As can be seen in Table 5.1, preservation of osteological structures at the microscopic level is generally poor for these samples. In fact, the majority of these samples show limited birefringence and some even lack recognizable histological features under polarized light (e.g., Civil War MM530 femur; see Plates 5.1 and 5.2).

Preservation of some samples is mixed within a single section and is most apparent when comparing internal to external areas of bone (e.g., Plate 5.3).

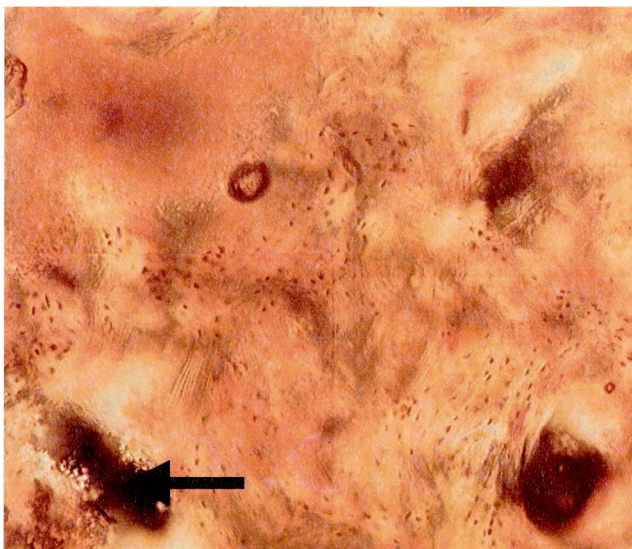


Plate 5.1: MM530. Poor preservation of histological structures. Dense and highly compact bone. The interior of bone contains a mixed bone matrix pattern. One can see empty lacunae (small dark spots), osteons (one with crystals or foreign material) (arrow), some lamellar bone and lots of woven bone. Poor preservation or high degree of mosaicism of bone is illustrated here. Note no Maltese cross of osteons. Photo taken from section A (see Plate A.1 in Appendix). Magnified in Photoshop by 50%.

Sample	Preservation		Syphilis Pathology	
	Histological Index (Millard, 2001)	Maltese Cross	Grenzstreifen	Polsters
Civil War MM530 femur	2	few	+?	-
UK HMC94-SK805	5	+	+?	+?
UK HMC94-SK932 right humerus	2	few	-	-
UK HMC94-SK932 long bone path	3	medium	- unobservable	-
UK HMC94- SK1121 fibula	4	+	+	+
UK HMC94- SK1121 tibia	4	+	-	+
UK HMC94- SK1216 tibia fragment 1	1	for those observable osteons	+?	+?
UK HMC94- SK1216 tibia (1b) fragment 2	1	-	-	-
UK SK77 rib	0	-	-	-
Belleville B302 femur	5	+	+	+?

Table 5.1: Summarized results from histological analyses.

Histological Index: 0=no original features identifiable, except Haversian canals may be present; 1=Haversian canals present, small areas of well-preserved bone present, or lamellate structure is preserved by pattern of destructive foci, 2=some lamellate structure is preserved between the destructive foci, 3=some osteocyte lacunae preserved; 4=bone is fairly well preserved with minor amounts of destructive foci, 5=very well preserved, virtually indistinguishable from modern bone. “few” = small number of observations. “+” = observable. “+?” = possible presence. “-” = none observed.

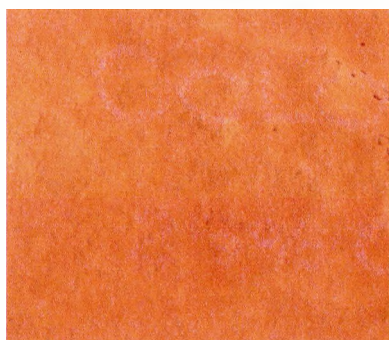


Plate 5.2: MM530. Lack of histological structures.

Interior or what should be the marrow space and/or trabecular bone. There are no recognizable structures due to poor preservation.

Photo taken from section B (see Plate A.1 in Appendix). Magnified in Photoshop by 33%.

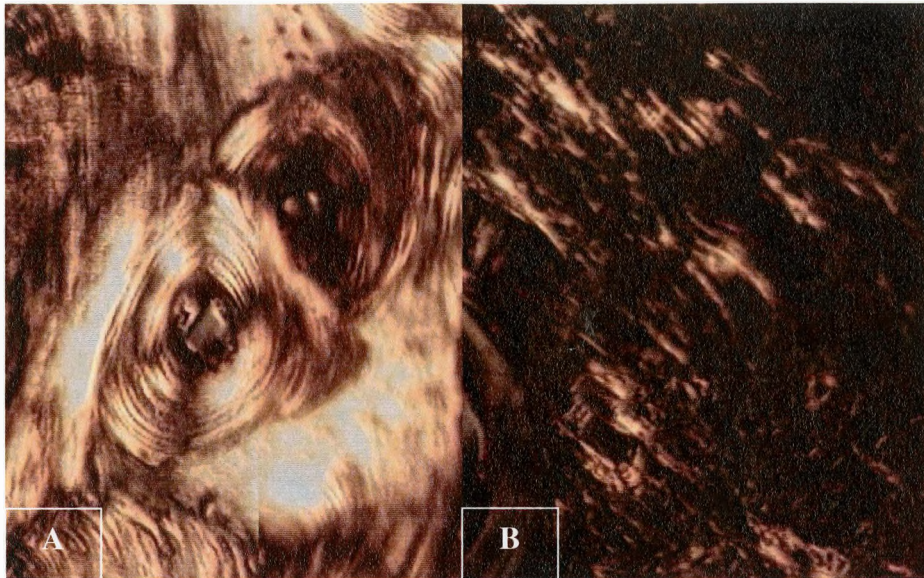


Plate 5.3: HMC94-SK1216 tibia fragment 1

Comparison of interior bone (A) vs. exterior bone (B). Note the difference in bone structure preservation. Osteons and birefringence apparent in photo A, but not in B. Magnified 67% (A – photo taken from section 8 interior; see Plate A.7 in Appendix for location) and 50% (B – photo taken from section 8 near periosteal surface) by Photoshop.

Only samples UK HMC94-SK805, SK1121 and Belleville B302 illustrate the presence of well preserved Haversian systems with lamellar bone, creating the Maltese cross under polarized light and lamellae within the different areas (i.e., interstitial, periosteal and endosteal) (see Plates 5.4, 5.5 and 5.6).

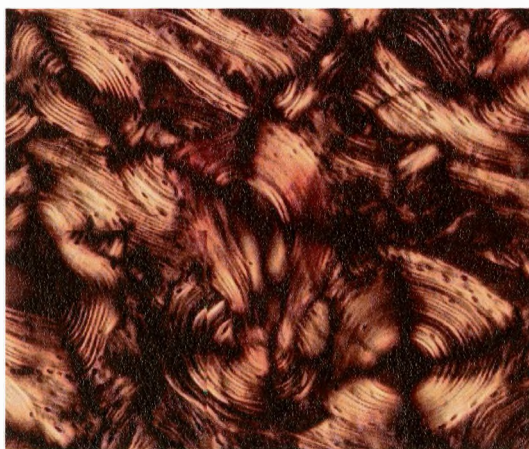


Plate 5.4: Belleville B302 femur

Well preserved histological structures showing clear birefringence of lamellar bone with the production of the Maltese cross in Haversian systems. This bone has undergone lots of turnover/remodeling evidenced by the increased number of osteons (and secondary osteons). Note the red/brown staining of unknown etiology of the one osteon in the center of the picture. Photo taken from bottom of macroscopic picture (see Plate A.10 in Appendix). Magnified 50% by Photoshop.

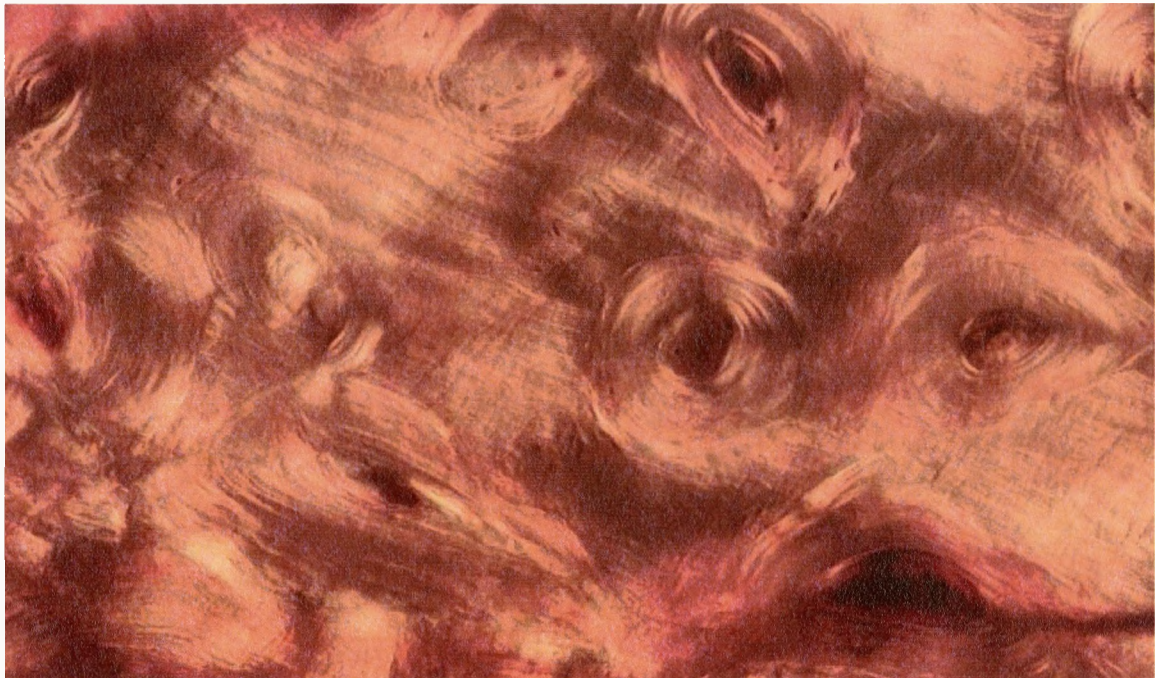


Plate 5.5: HMC94-SK1121 tibia.

Illustrates preservation of Haversian Systems and lamellae. More interstitial lamellae than osteons are observed, therefore, bone deposition is more prominent than remodeling. Photo taken from section A interior (see Plate A.6 in Appendix for location). Magnified 33% by Photoshop.

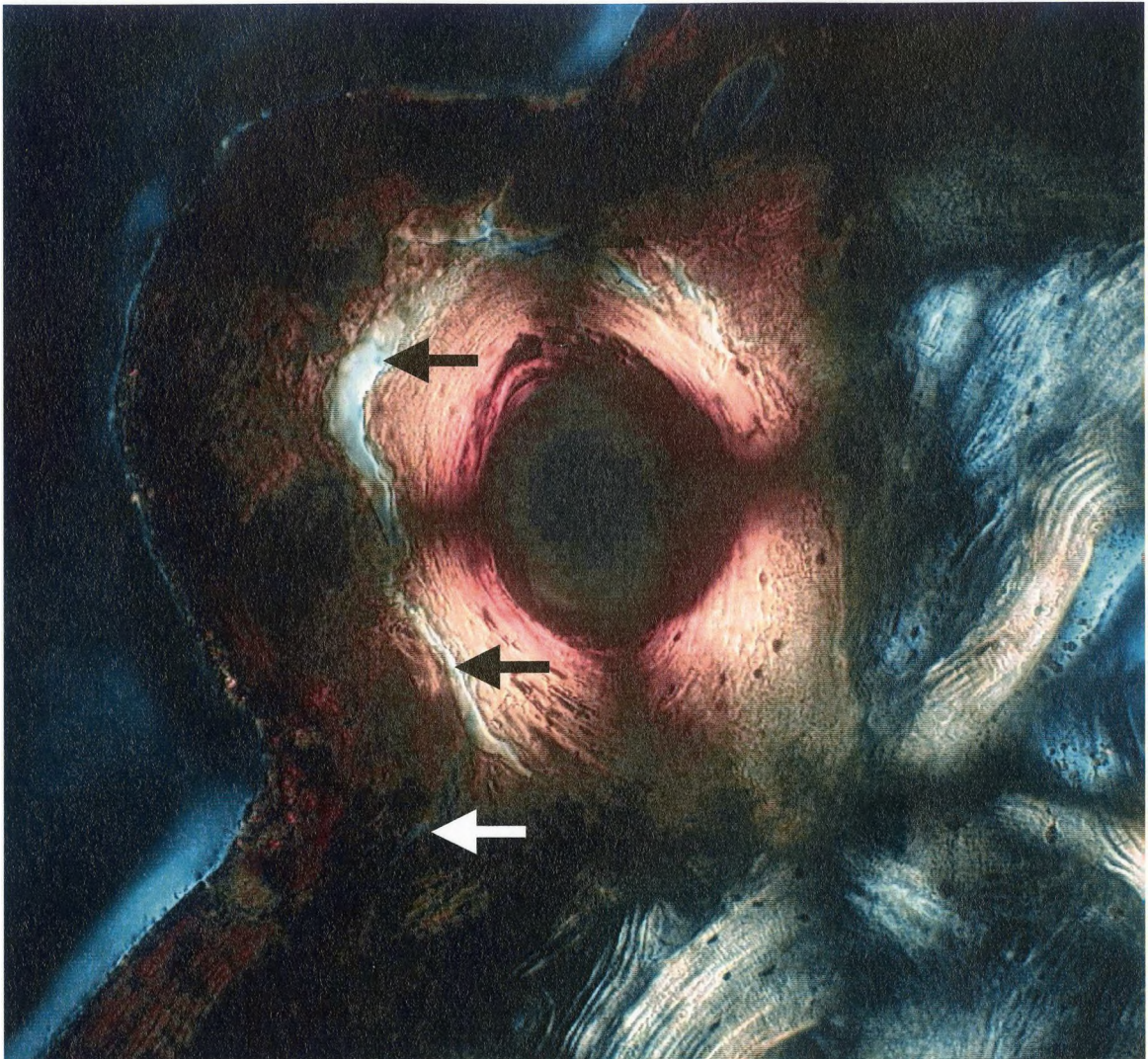


Plate 5.6: HMC94-SK805 fibula. Illustration of Maltese Cross in Haversian System and lamellar bone.

This may represent the presence of a Polster with a Grenzstreifen structure. One can see that the periosteum is breaking away from the Haversian System which could be the result of the embedding/cutting procedure (arrows). There are several instances of this layer cracking along the exterior, in plane with the osseous structures (arrows), which helps to illustrate the different nature/composition of the bone.

Note the presence of numerous osteons, especially close to the periosteum, which shows turnover of bone and longevity of osseous activity (suggesting a chronic state).

Photo taken from section 1 (see Plate A.2 in Appendix for location).

Magnified 67% by Photoshop and taken under Tungsten light.

Specific alterations caused by diagenesis are observed for several samples. For example, microorganism focal destruction in the form of Wedl canals (described by Hackett, 1981) is observed in both samples of UK HMC94-SK932 (see Plates 5 7-5.9) and in UK HMC94-SK1121 fibula (see Plate 5 10).

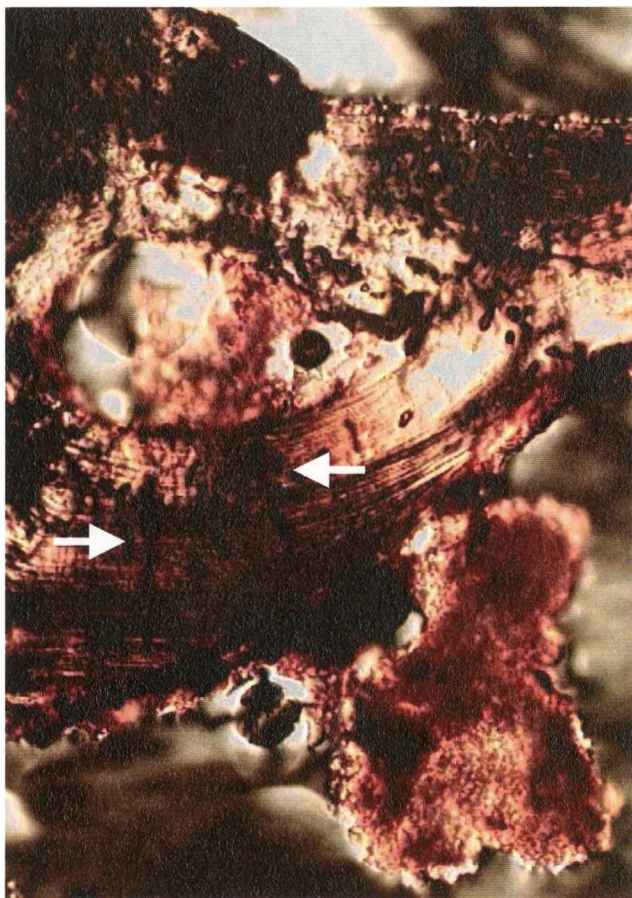


Plate 5.7:

HMC94-SK932 proximal humerus. Presence of Wedl canals (arrows) running through lamellar bone in area of the beginning of trabecular bone. Note presence of foreign substances in the Haversian System. Lamellar bone is noticeable, but exhibiting poor preservation. Photo taken from section 3 middle (see Plate A.3 in Appendix for location). Magnified 50% by Photoshop.

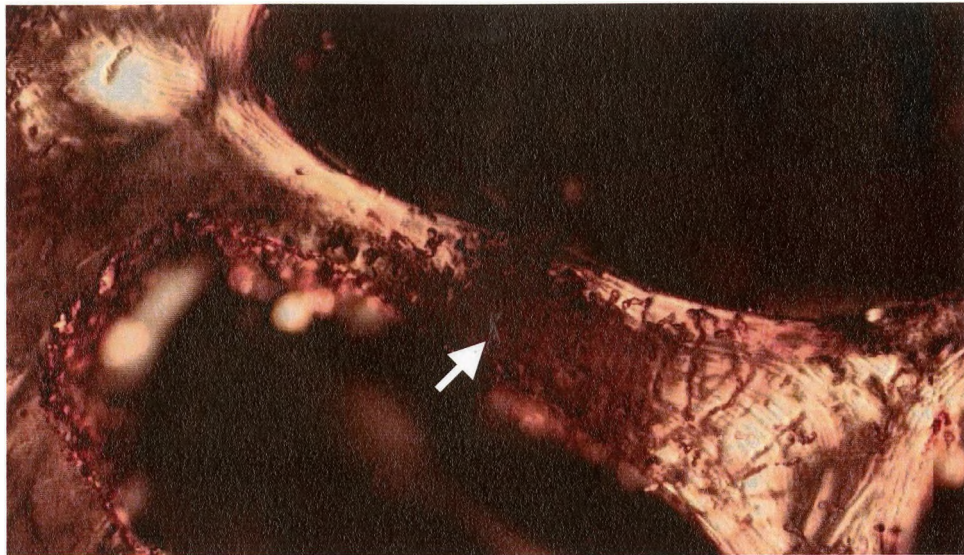


Plate 5.8: HMC94-SK932 proximal right humerus 2. Poor preservation of osseous structures still present. Wedl canals, foreign (fungal) (arrow) material and little lamellar bone in trabecular area can be observed. Cut several mm interior to previous section with this picture being taken from section 1 (see Plate A.3 in Appendix). Magnified 50% by Photoshop.



Plate 5.9: HMC94-SK932 long bone path. Once again this histological sample shows poor preservation of osseous structures as only small areas of lamellar bone can be identified. Fungus or another microorganism is filling in the bone. Wedl canals (W) are observed as well as foreign substances in the Haversian System (arrow). Photo taken from section 7 (see Plate A.4 in Appendix). Magnified 33% by Photoshop.



Plate 5.10: HMC94-SK1121 fibula.

Illustration of Wedl canals that were found in the cortical bone. Although these structures were present, they were quite sparse in comparison to the other histological sections. Photo taken from section 5 interior (see Plate A.5 in Appendix for location). Magnified 50% by Photoshop.

Also, foreign materials (e.g., soil particulates, crystals) within lacunae and/or Haversian systems are seen in all samples (e.g., see Plates 5.1, 5.11 [MM530-3], Plates 5.8, 5.9 [HMC94-SK932], Plate 5.10 [HMC94-SK1121], Plates 5.12, 5.13 [HMC94-SK1216], and Plate 5.14 [SK77]). Fungal and/or microorganism intrusion of unknown origin is also present in several samples (see Plates 5.8, 5.9 [HMC94-SK932], and Plate 5.15 [HMC94-SK1121]).



Plate 5.11: MM530.

“Swiss Cheese” appearance of bone caused by lytic processes in osteitis. The lower left corner is the exterior of the cortical bone. Upper right is an example of a foreign substance in the large Haversian canal (in circle).

Photo taken from area C (see A.1 in Appendix for location).

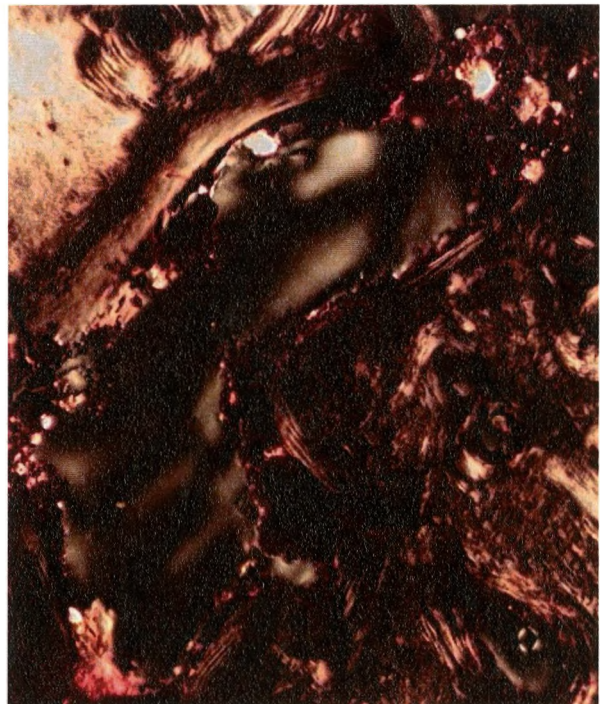
Magnified in Photoshop by 5%.

Plate 5.12: HMC94-SK1216 tibia fragment 1

A very large lacuna/hole with foreign material (crystal-like). Note the presence of little lamellar bone, but presence of either woven bone or poorly preserved bone. This represents another illustration of osteitis.

Photo taken from section 7 (see A.7 in Appendix for location).

Magnified 50% by Photoshop.



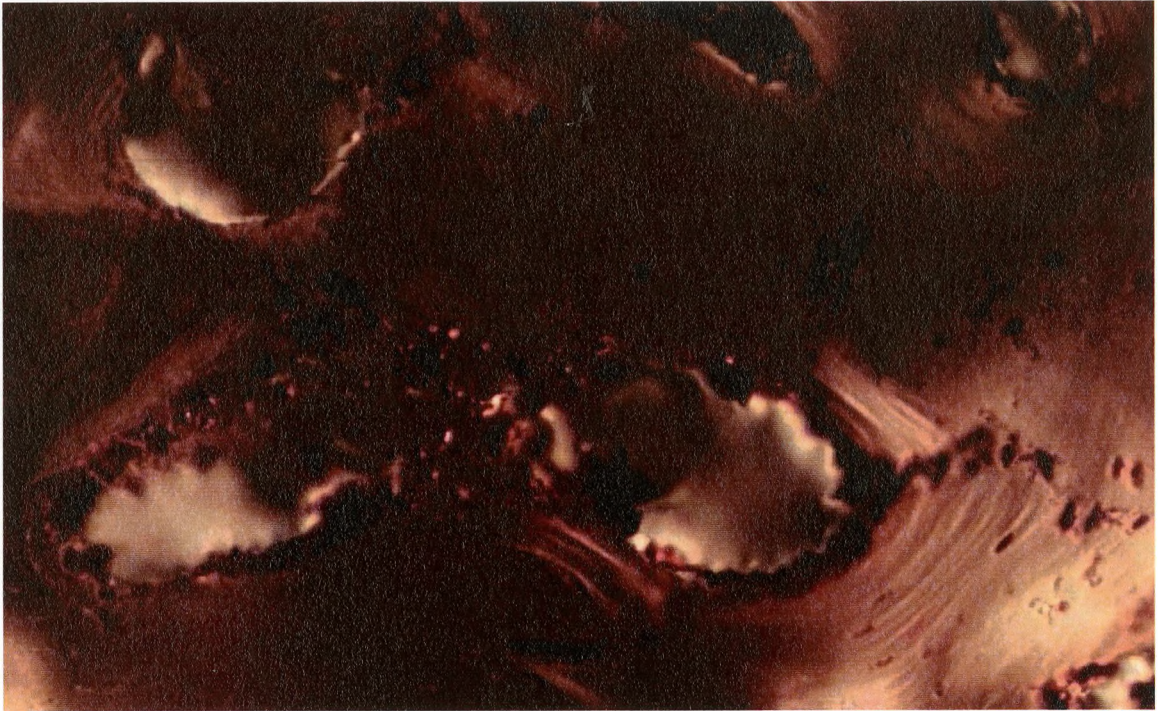


Plate 5.13: HMC94-SK1216 tibia fragment 2.

This section shows that very minute amounts of lamellar bone are observable due to the poor preservation of histological structures. Note the presence of foreign material in the lacunae/osteons.

Photo taken from section 4 interior (see Plate A.8 in Appendix for location). Magnified 67% by Photoshop.

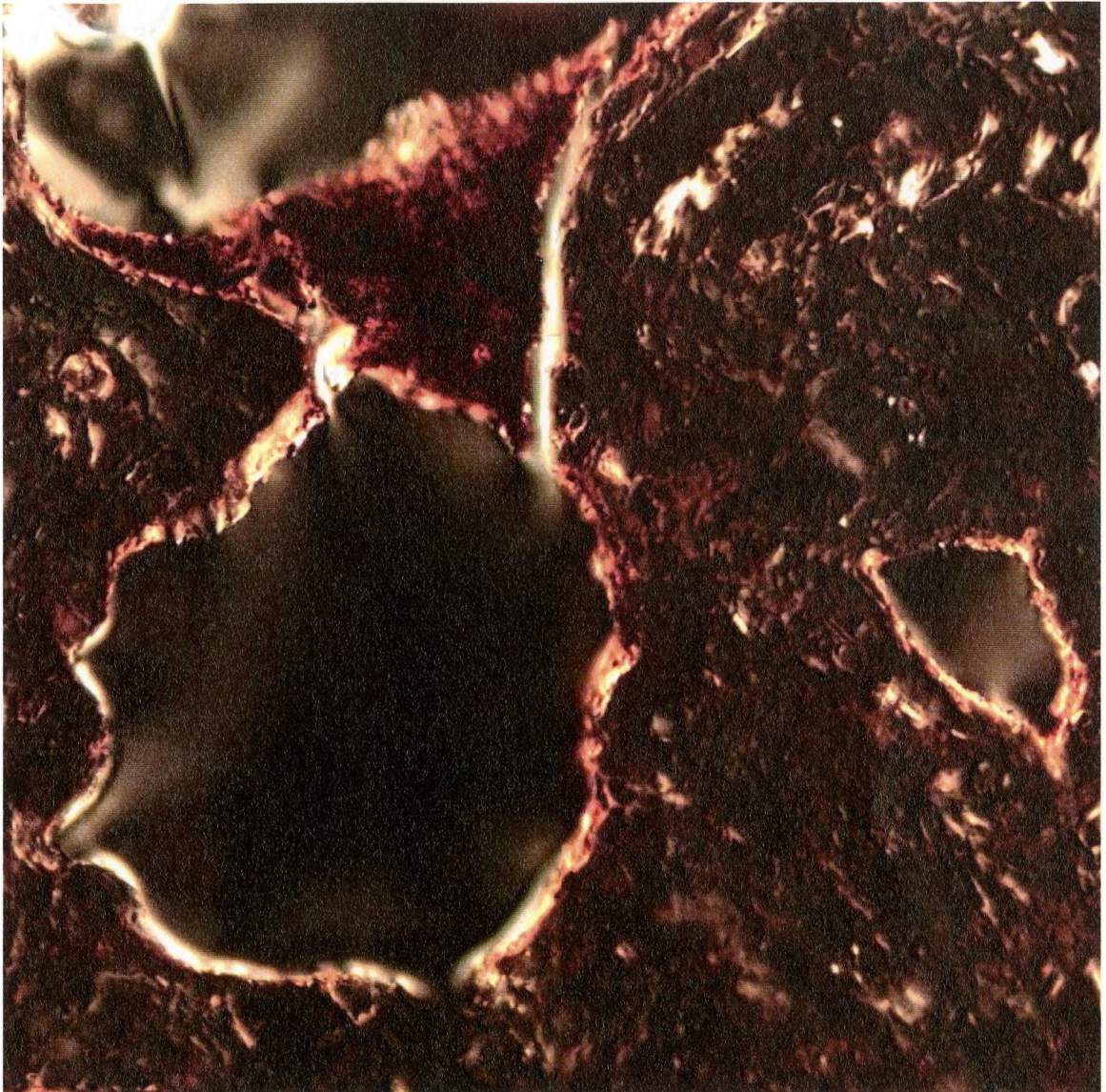


Plate 5.14: Blackfriars SK77

There is extremely poor preservation of histological structures (i.e., no lamellae, Maltese cross) with the presence of foreign red/brown material in the open spaces throughout this entire section. Note the irregular border of the large lacuna/open space possibly due to lytic processes or the proliferation of bone.

Photo taken from section 1 interior (see Plate A.9 in Appendix for location). Magnified 50% by Photoshop.

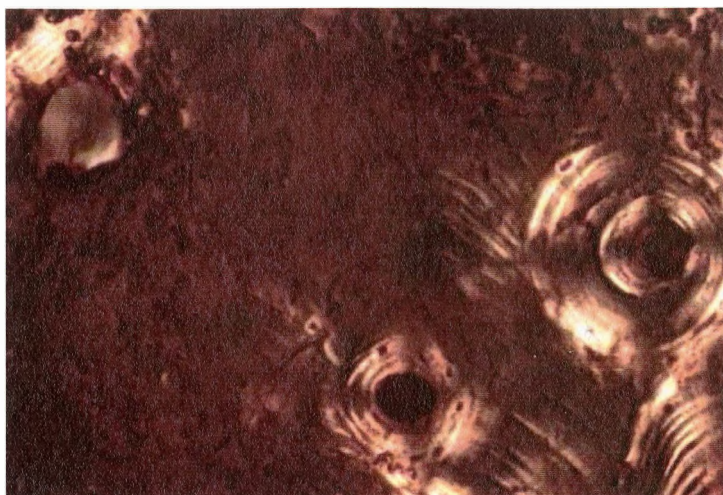


Plate 5.15: HMC94-SK1121 fibula.

Illustration of a small area showing poor preservation of osseous structures and possible fungal inclusion.

Photo taken from section 6 interior (see Plate A.5 in Appendix for location). Magnified by Photoshop 25%.

Red/brown staining of the surfaces, both endosteal and periosteal (more prevalent), is observed to some extent in all samples from the Hull Magistrates Court (HMC94) site (see Plate A.2 in Appendix, Plates 5.16, 5.17 [HMC-SK805], Plate 5.18 [HMC94-SK932], Plates A.5 in Appendix, Plate 5.30 [HMC94-SK1121]) which makes observations of the periosteal surface difficult to record. This external staining is most striking in samples UK HMC94-SK805 and HMC94-SK1121 which consequently have the best preservation of histological structures for this archaeological site. A difference in colouration between the endosteal and external areas of bone is also observed for Civil War MM530 femur (see Plate A.1 in Appendix).



Plate 5.16: HMC94-SK805 fibula.

Example of staining on the exterior of the cortical bone. Note the undulating surface of the periosteum. This may be a Polster structure.

Photo taken from section 4 (see Plate A.2 in Appendix for location).

Magnified 17% by Photoshop.

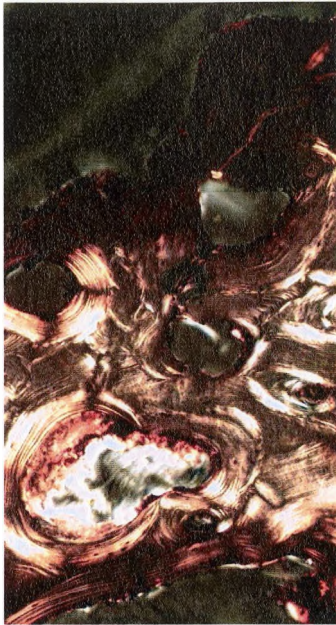


Plate 5.17: HMC94-SK805 fibula.

Picture shows endosteal and periosteal surfaces. Note red/brown staining on both surfaces, that are however, more prominent on the exterior of the bone. This has made it difficult to observe structures in this area. One can observe a foreign substance in the large Haversian system. The presence of osteitis is illustrated by the large lytic lacunae.

Photo taken from section 3 4 (see Plate A.2 in Appendix for location).

Magnified in Photoshop at 33%.



Plate 5.18: HMC94-SK932 proximal right humerus.

Remnants of a Maltese Cross and lamellar bone in the one Haversian System, but then observe the osteons to the right of this which do not have such characteristics. Bone is poorly preserved due to the lack of recognizable structures. Note the presence of slight red/brown staining on the surface of the cortical bone. Photo taken from section 1 (see Plate A.3 in Appendix for location). Magnified 50% by Photoshop.

As previously mentioned, the lack of good osseous preservation has led to some difficulties in discerning microscopic structures indicative of syphilis. However, several samples provide possible histological bone presentations that point to chronic, episodic, osteoclastic and osteoblastic processes characteristic of syphilis. For example, Grenzstreifen and/or cement lines separating the original compact bone from the newly built bone is tentatively observed in Civil War MM530 (Plates 5.19 and 5.20), UK HMC94-SK805 fibula (Plate 5.6) and SK1216 tibia fragment 1 (Plate 5.21), but is confidently identified in UK HMC94-SK1121 fibula (Plates 5.22 and 5.23) and Belleville B302 femur (Plate 5.24).

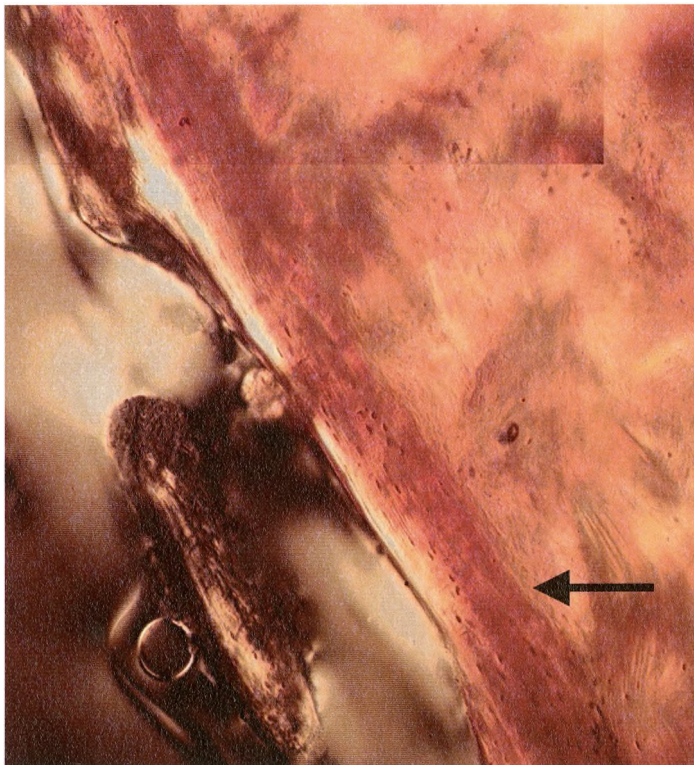


Plate 5.19: MM530.

Lamellar bone, the remnant of an osteon and the mosaic pattern of the bone matrix are evident.

There is a cement line (arrow) on the edge of the cortical bone which represents a possible Grenzstreifen structure. Bone is lamellated and organized in this section.

Photo taken from section D (see Plate A.1 in Appendix for location).
Magnified 17% in Photoshop.



Plate 5.20: MM530.

Here, layers of bone are separate from the cortex.
Note the cement line on the outer surface of the bone
(arrow). On the outer extremity the bone is darker in
colour with no organized structures.

Photo taken from section E (see Plate A.1 in
Appendix for location).
Magnified by 25% Photoshop.

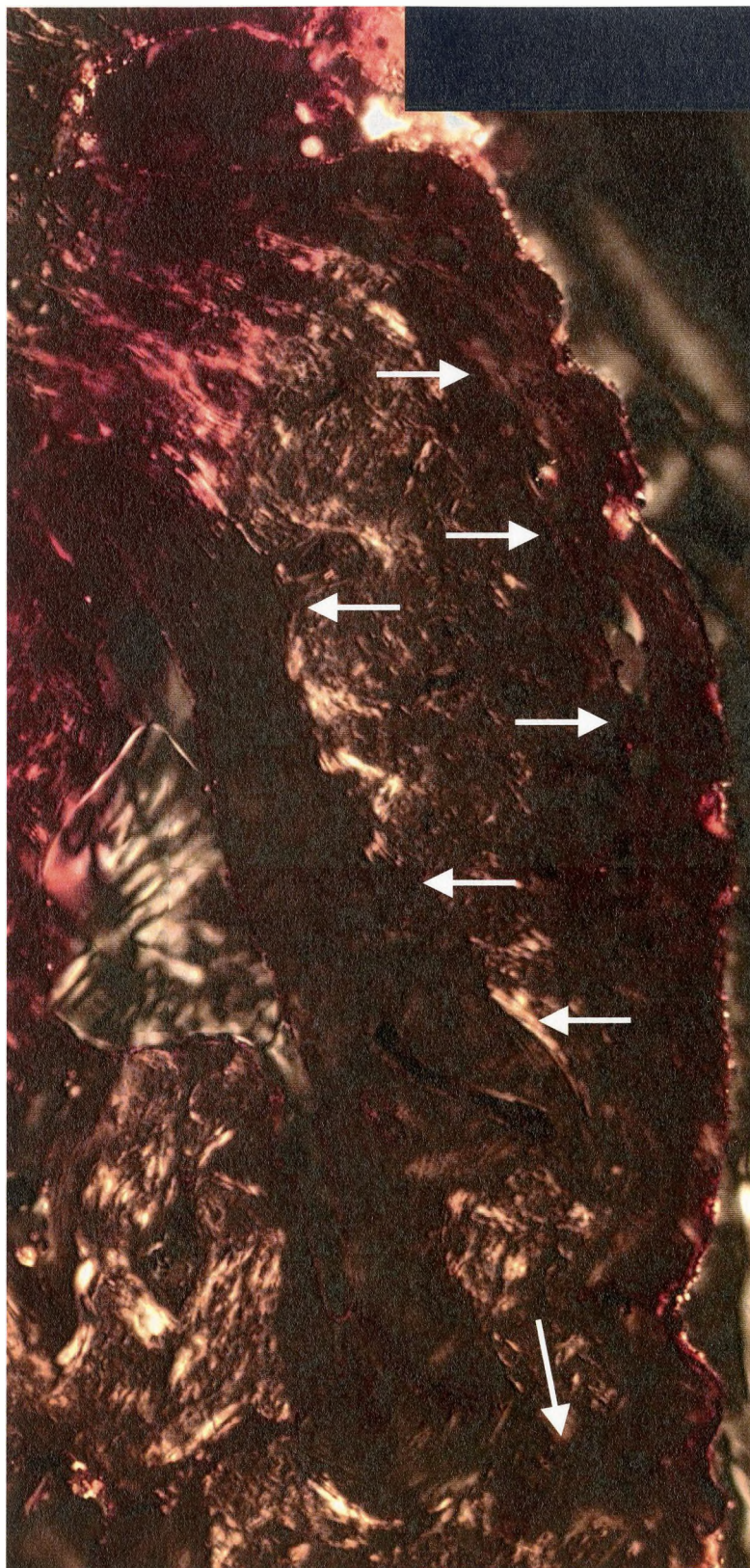


Plate 5.21: HMC94-SK1216 tibia fragment.

Illustrates a difference in bone growth as distinct layers can be seen (arrows).

Photo taken from section 1 (see Plate A.7 in Appendix). Magnified 17% by Photoshop.

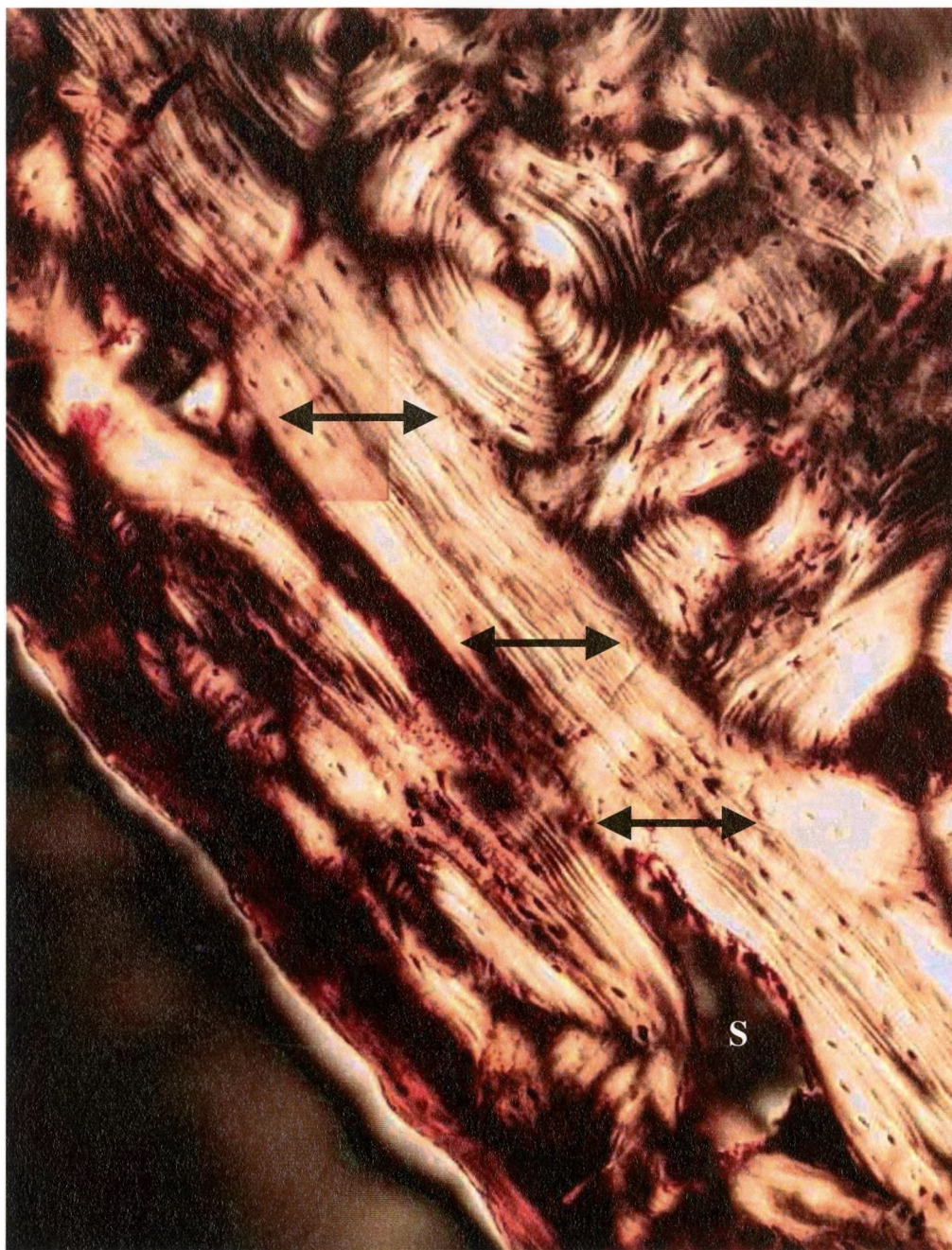


Plate 5.22: HMC94-SK1121 fibula. Observation of good preservation of osseous structures; presence of Haversian Systems with Maltese Cross and lamellar bone (intra and interstitial). Sinous lacunae (S) are also illustrated between original compact bone and newly built bone. Grenzstreifen and/or a cement line demarcates the original periosteum from new bone proliferation (arrows). New bone is of a chronic nature due to the observation of bone turnover (osteons). Note red/brown staining on exterior of bone. Photo taken from section 1 (see Plate A.5 in Appendix). Magnified 17% by Photoshop.



Plate 5.23: HMC94-SK1121 fibula.

Another illustration of the Grenzstreifen or cement line (arrows) demarking original from newly built bone on the exterior of the shaft. Foreign material is observed in several of the Haversian Systems. Observation of large lytic lacunae (L) represent the presence of osteitis. Photo taken from section 1 (see Plate A.5 in Appendix) just superior to previous picture. Magnified 17% by Photoshop.

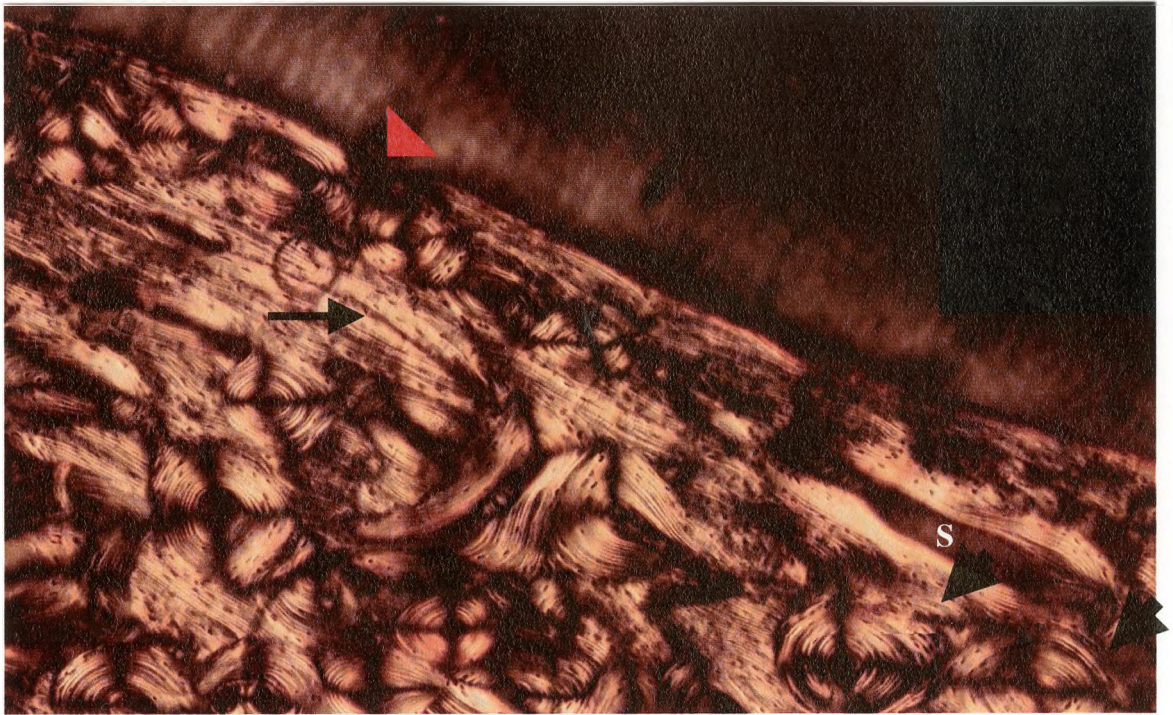


Plate 5.24: Belleville B302 femur

Possible Grenzstreifen near periosteal surface of shaft. One can observe two different episodes of bone deposition (arrow) within this structure. There is an indentation at the periosteal surface (triangle) for a vascular structure or possibly the beginning/ending of a Polster. Possible old Polsters are filled in just interior to Grenzstreifen in the lower right of picture (fat arrows). A sinus lacuna (S) is found on the right of the picture situated within the Grenzstreifen.

Photo taken from bottom of macroscopic picture (see Plate A.10 in Appendix).

Magnified 33% by Photoshop.

Sinus lacunae are also identified within the Grenzstreifen for both UK HMC94-SK1121 fibula and Belleville B302 (see plates mentioned above). Polsters are also tentatively observed in UK HMC94-SK805 (Plates 5.6 and 5.25), SK1216 tibia fragment 1 (Plate 5.26) and Belleville B302 (Plates 5.24 and 5.27), but are confidently identified in UK HMC94-SK1121 fibula (Plates 5.28 and 5.29) and tibia (Plates 5.30-32). Those Polsters identified in UK HMC94-SK1121 bone samples contain densely packed lamellae indicative of this structure.



Plate 5.25: HMC94-SK805 fibula.

Two possible Polsters with Haversian Systems in the middle.

Photo taken from section 1 (see Plate A.2 in Appendix) and just superior to that shown in Plate 5.6.

Magnified 33% by Photoshop and taken under Tungsten light.

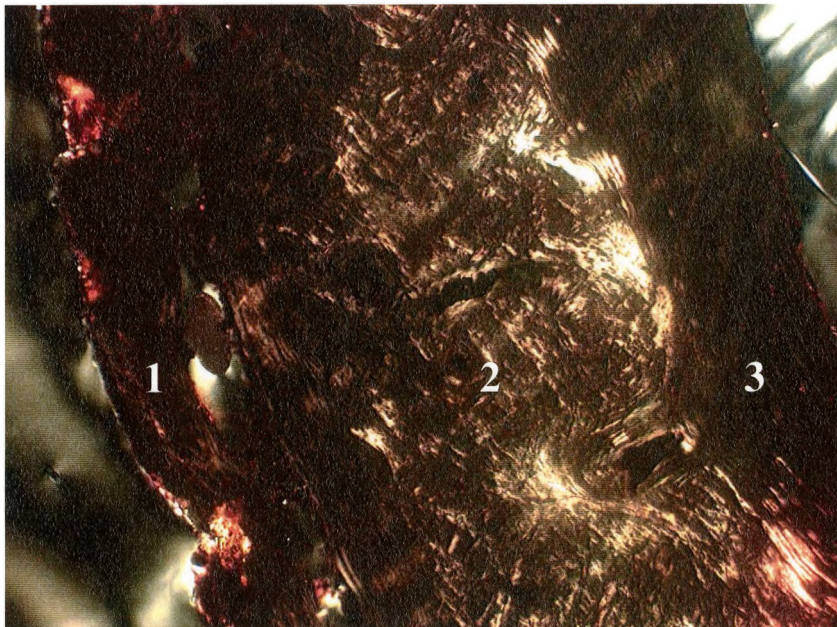


Plate 5.26: HMC94-SK1216 tibia fragment.

Close up of Plate 5.21 to better illustrate the differences (as in stages) in bone proliferation – possibly three different layers (labeled 1-3). Layer 1 appears to have shallow, elongated Polster-like structures. Magnified 50% by Photoshop.

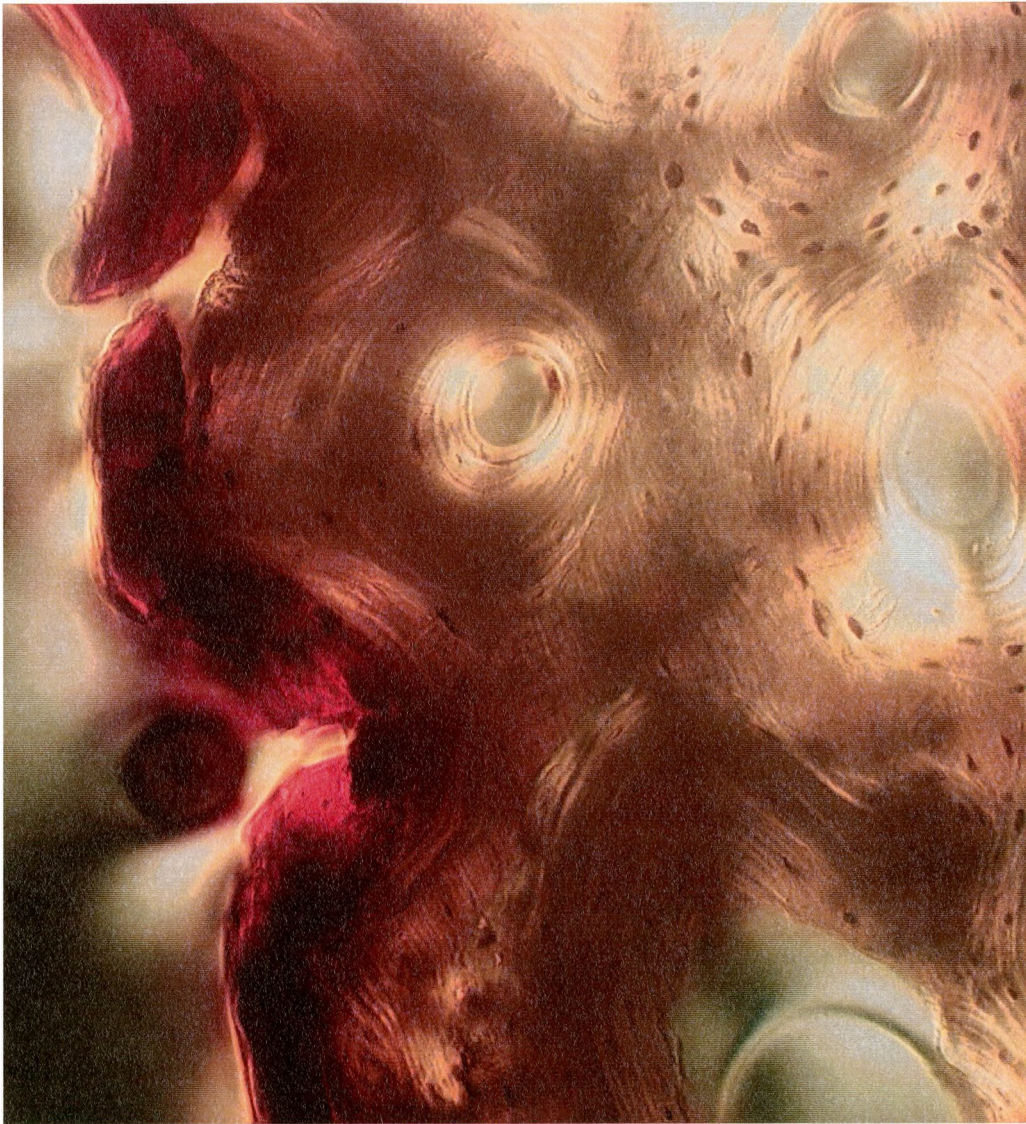


Plate 5.27: Belleville B302 femur

The possible presence of a small Polster that has been remodeled.
The red line is from another researcher marking the surface of the bone in previous years.
Photo taken at the anterior portion of femoral shaft (see Plate A.10 in Appendix).
Magnified 50% by Photoshop.

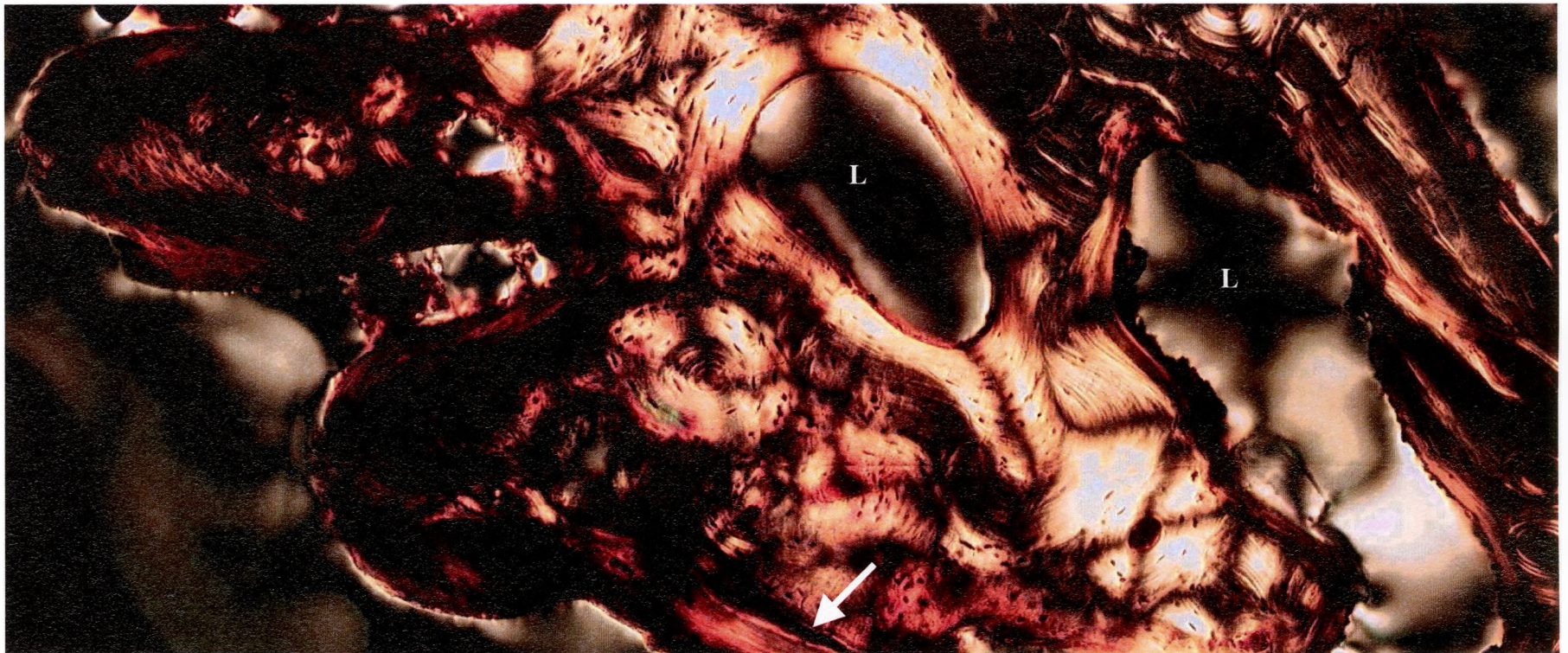


Plate 5.28: HMC94-SK1121 fibula.

Presentation of Polsters filled with dense lamellae. Also see Haversian Systems within these Polsters which suggests a chronic situation as bone has been able to remodel. Near the bottom middle of picture one can see the Grenzstreifen formation (arrow). Large lytic lacunae (L) represent the presence of osteitis.

Photo taken from section 3 (see Plate A.5 in Appendix for location). Magnified 17% by Photoshop.

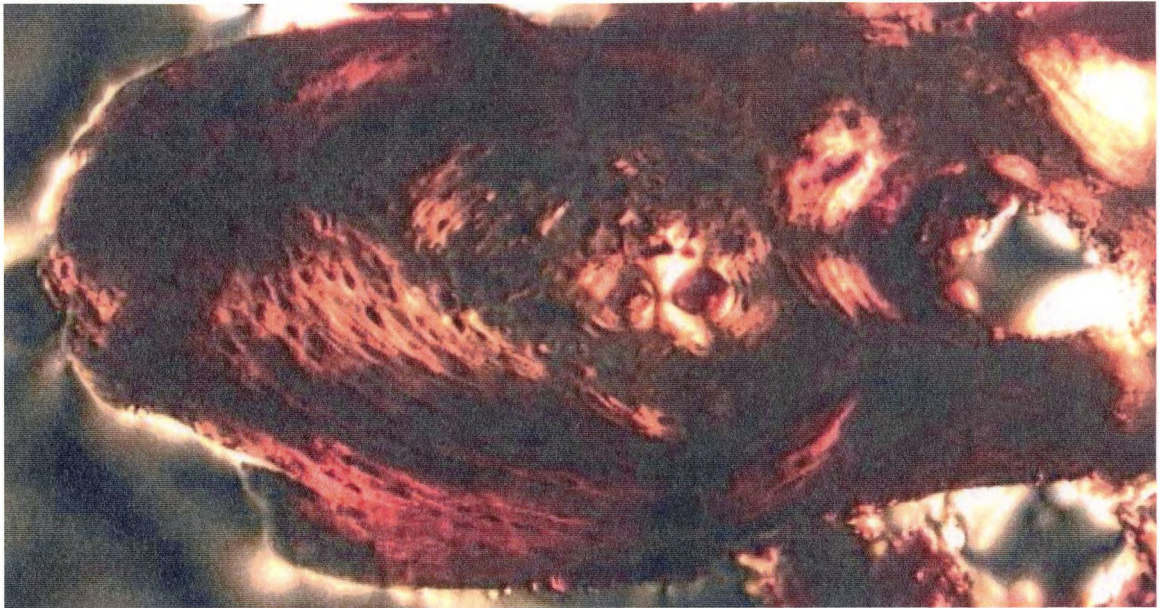


Plate 5.29: HMC94-SK1121 fibula.

Close up of upper Polster from previous picture illustrating dense lamellae (and Haversian System). Magnified 50% by Photoshop.



Plate 5.30: HMC94-SK1121 tibia.

Illustrates Polsters with both lamellae (dense sections closer to external edges of bone) and osteons, therefore signifying a chronic state. Note the red/brown staining on exterior of bone (especially to the right, as the left is red marker put on by the author). Photo taken just exterior to Plate 5.5. Magnified 17% by Photoshop.



Plate 5.31: HMC94-SK1121 tibia.

Illustration of a possibly very large Polster (but not rounded as it could be constrained by vascular structures). Lots of quickly laid down lamellar and woven bone with external edges densely packed with lamellar bone. Note at the top middle of the picture it appears that there is a Polster-like structure (see Plate 5.32) that has been ‘filled in’ and no longer exhibits the pillow-like formation. This could represent what happens in some stages of chronicity whereby Polsters have developed long ago, but remodeling has already started to fill in the ‘gaps’ Photo taken from section C (see Plate A.6 in Appendix for location). Magnified 33% by Photoshop.

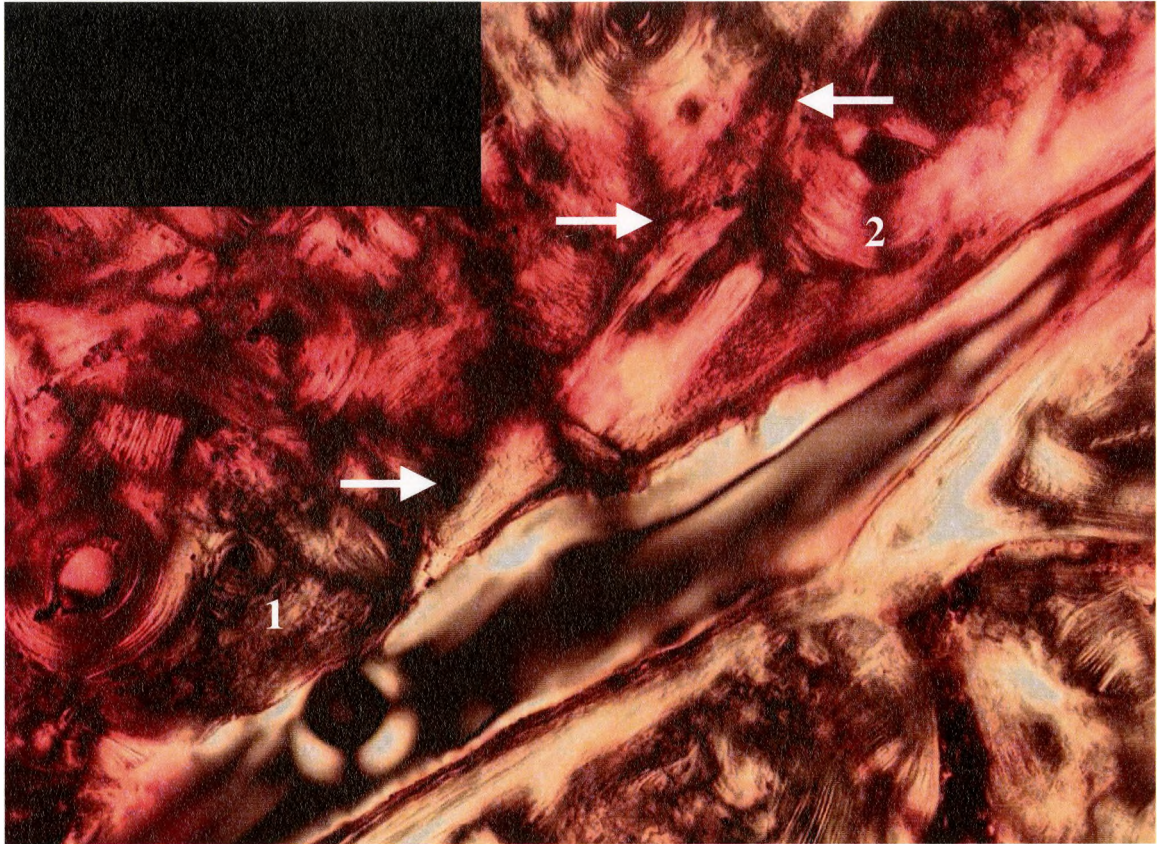


Plate 5.32: HMC94-SK1121 tibia.

This picture incorporates the last picture from the top and expands to the right to show a better view of possible remodeled Polsters (labeled 1 and 2). There is new lamellar and woven bone which has filled in the gap between the two structures. One can also see the 'cement line' from original to new bone growth (arrows). Magnified 25% by Photoshop.

For those sections of bone that came directly from macroscopically apparent pathological regions (this was not possible for Belleville B302), their histology reveals some general trends indicative of abnormal processes caused by inflammation. Areas of focal destruction (i.e., osteoclastic) are observed in all specimens (e.g., see Plates 5.3 [MM530], Plate 5 17 [HMC94-SK805], Plate 5 12 [SK1216], Plate A.9 in Appendix

[Blackfriars SK77], and Plates 5.33-35 [Belleville B302]) and UK HMC94-SK932 long bone path (Plate 5.36) and SK1216 tibia fragment 2 (Plate 5.37) show excellent examples of the mixture of both destructive and proliferative processes known to syphilis.

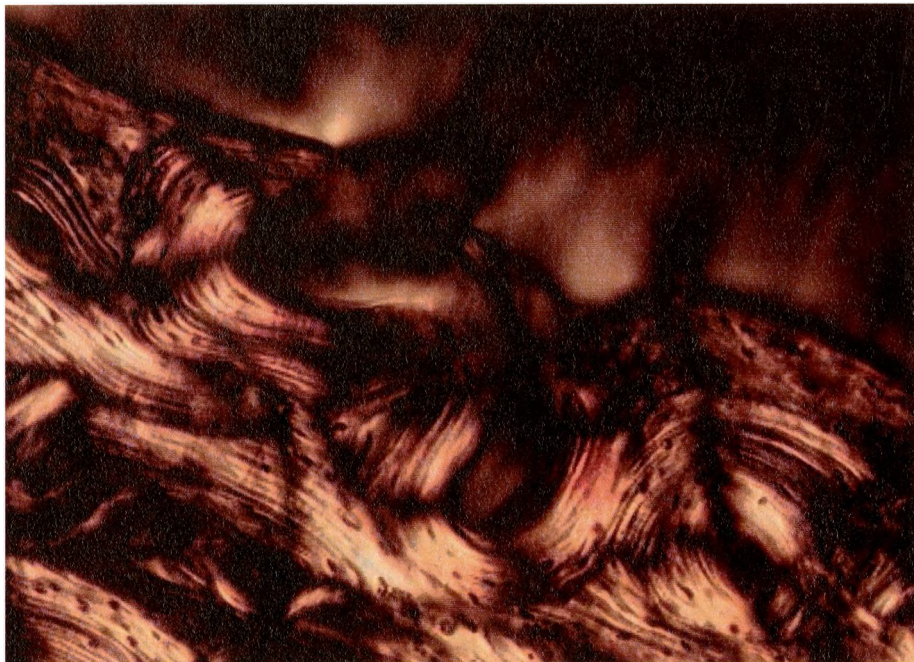


Plate 5.33: Belleville B302 femur

Periosteal surface illustrating lytic/destructive areas or possibly post mortem damage. Photo taken from bottom of macroscopic picture (Plate A.10 in Appendix for location). Magnified 67% by Photoshop.

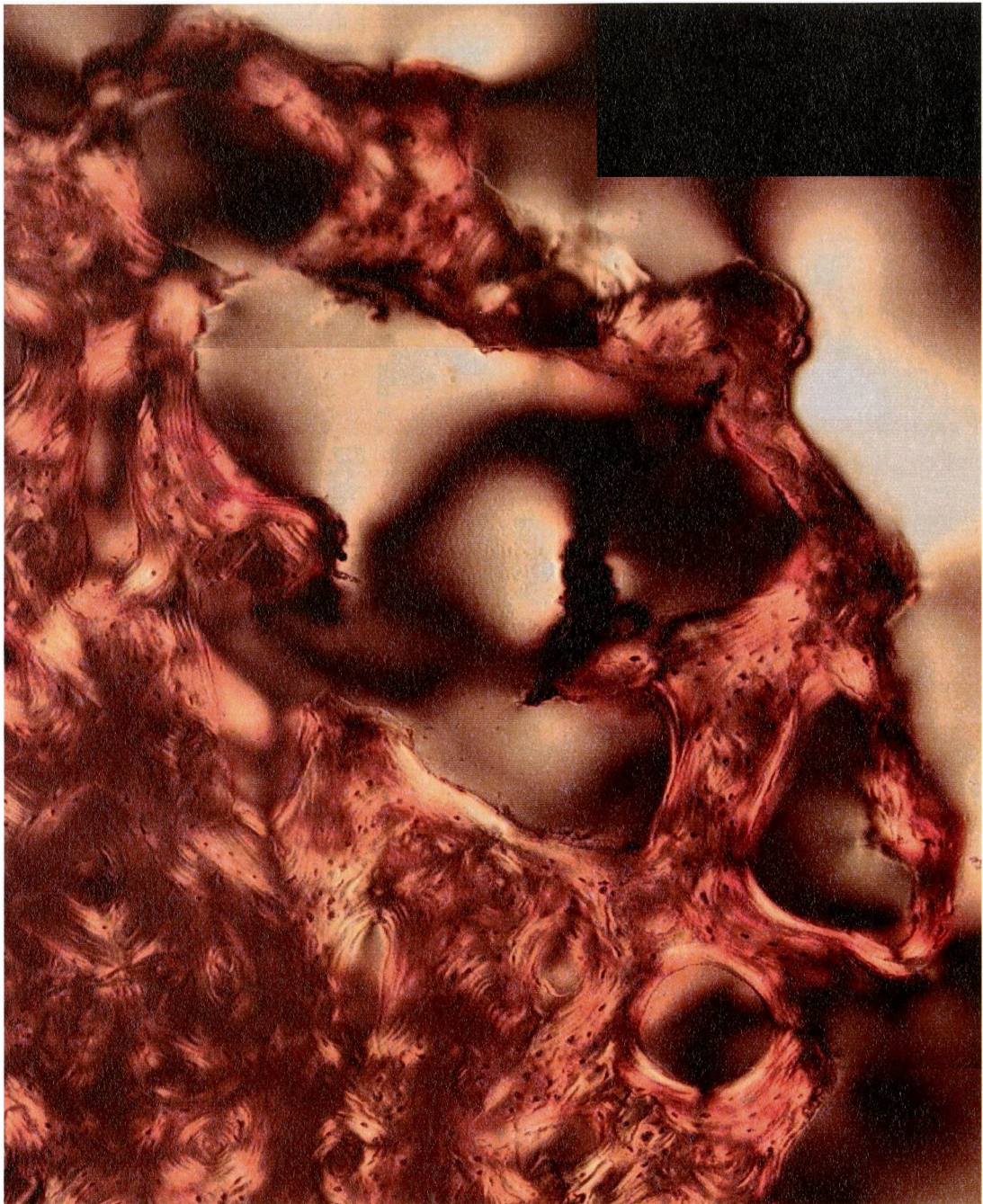


Plate 5.34: Belleville B302 femur

Bone not as well preserved in this region. Large lytic areas caused by osteitis with little bone deposition suggesting this disease is in an active phase within this area. Photo taken at linea aspera – no cover slip here because bone was too large (See Plate A.10 in Appendix for location). Magnified 25% by Photoshop.

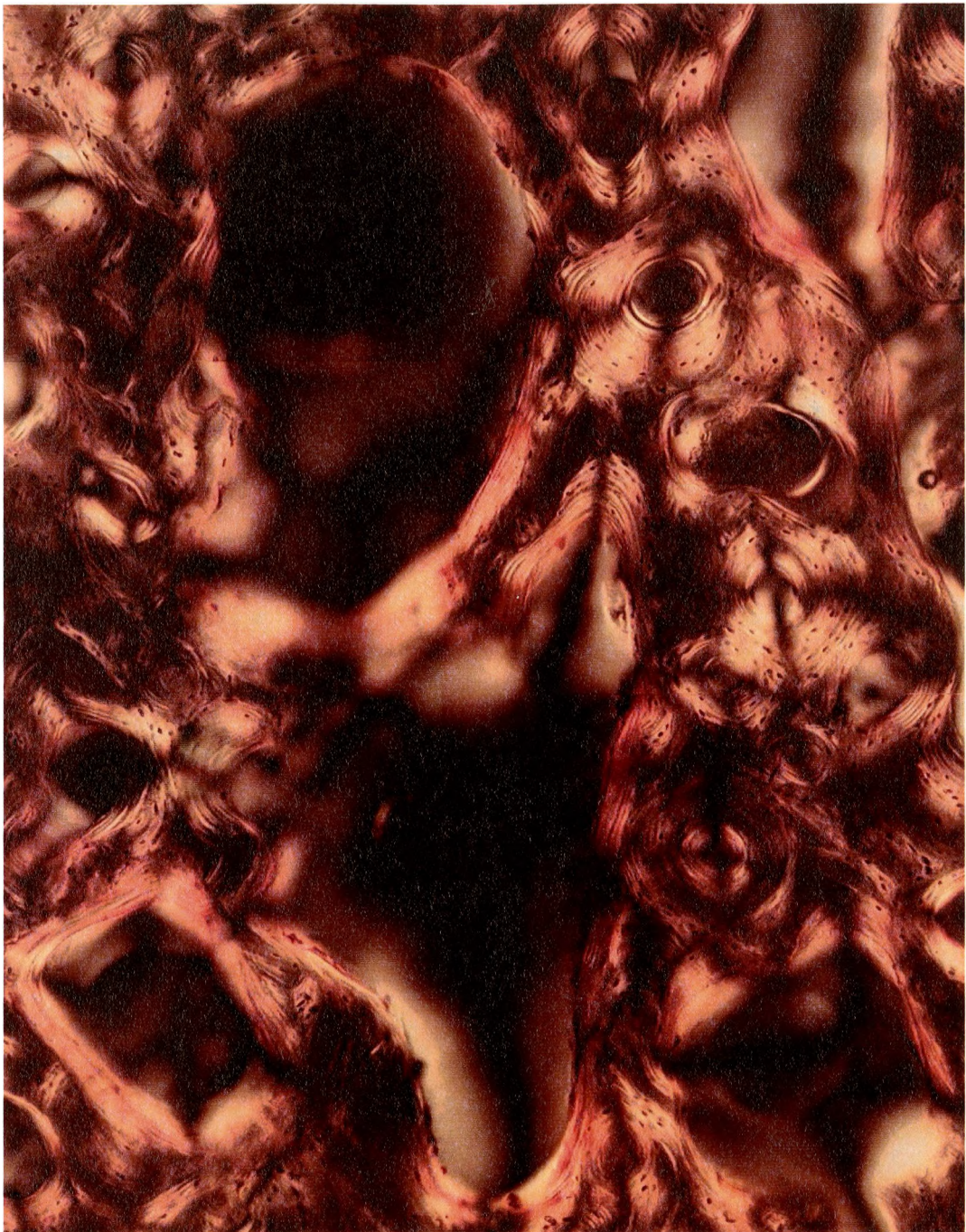


Plate 5.35: Belleville B302 femur

Illustration of large lytic lacunae just interior to the periosteum.

Photo taken at the frontal/anterior portion of femoral shaft (see Plate A.10 in Appendix for location). Magnified 25% by Photoshop.



Plate 5.36: HMC94-SK932 Long bone pathological. This illustrates proliferative bone growth in a fan-like and layered presentation from the cortical/original bone (right side of picture). Macroscopic observation of pathology (heavy periostitis) is found in this area. This is only seen in this area of the bone. In the cortex, it is possible to see a few osteons with the Maltese Cross. Photo taken from section 8 (see Plate A.4 in Appendix for location). Magnified 8% by Photoshop.



Plate 5.37:
HMC94-SK1216
tibia fragment.

View to show
lytic/destructive
areas and excess
formation of bone
proliferation caused
by osteitis.

Photo taken from
section 4 (see Plate
A.8 in Appendix for
location).
Magnified 8% by
Photoshop.

5.2 Molecular Analyses

Molecular results obtained from the samples studied in this project are summarized in Table 5.2. It should be noted that all extraction and PCR blanks were negative for all amplification reactions.

5.2.a *mtDNA*:

A 281bp section of the first hypervariable region (HVRI) of human mitochondrial DNA was successfully amplified from all 16 individuals (e.g., see Figure 5.1) except for two: Civil War MM1622 and UK Whithorn healed gumma. Although mtDNA was amplified from the bone dust of Civil War MM530, both the long bone and femur did not provide mtDNA results. Upon further analysis, the sequence obtained from MM530 must be discounted as it matches one of the samplers, DY (see Table 5.3). Therefore, three of the 16 individuals were unsuccessful or an 81% (13/16) success rate is achieved for this type of DNA amplification. It is worth noting that the mtDNA amplicons from the samples used in this project matched neither the researcher, the samplers (DY [with the exception of Civil War MM530 bone dust matching] and SRS), other lab members (JLB), nor the positive control (K562). This ensures that contamination of the samples was not an issue while in the McMaster laboratory.

	Origin	Sample #	Bone Element	Non/Pathological	mtDNA	Morphological Sex	DNA Sex
1	Civil War, USA	1001949 MM2880-2	articular surface	np	mt2		nr
			bone dust (no decontamination)	?	2-1		nr
2		1001949 MM2880-4	articular surface	np	mt2		nr
			bone dust (no decontamination)	?	mt2		nr
3		1002700 MM1015	fibula end	np	mt2	M	M?
4		1001245	rib	np	mt2		nr
5		1002814 MM1154	skull?	p?	2-1	M	nr
			skull	p (healed?)	mt2		M?
			skull	p?	2-1		nr
6		1000304 MM1622	long bone	np?	nr		nr
			long bone with lots of spongy	np?	nr		nr
			bone dust with foil particles	?	nr		M
7		1002422 MM530	long bone (femur?)	p	nr	F?	nr
			femur in foil	p	nr		nr
			bone dust (no decontamination)	?	2-2		M
8		295209	skull (shellac)	np	mt2	F	nr
9		1000873 MM2510	skull	np?	mt2		3M
10	Hull, England	HMC94-SK805	fibula	p	mt2	M	nr
			long bone	np	mt2		3M
11		HMC94-SK932	humerus	p	2-1	M	F?
			tooth	np	2-1		3M
			long bone	p	mt2		M
12		HMC94-SK1121	fibula	p	2-2	F	nr
			right metatarsal	np	mt2		nr
			tibia	p	2-2		F
13		HMC94-SK1216	scapula	np	2-1	M	nr
			long bone	p	mt2		nr
			tibia	p	mt2		nr

Continued...

	Origin	Sample #	Bone Element	Non/Pathological	mtDNA	Morphological Sex	DNA Sex
14	<i>Whithorn, England</i>		healed? gumma	p	nr		nr
15	<i>Blackfriars, England</i>	SK77	rib	p	mt2	F	F
16	<i>Belleville, Canada</i>	B302	proximal radius	np	2-1	M	M
			mid femur	p	mt2		3M

Table 5.2: Sample summary of DNA results.

NP and P represent nonpathological and pathological bone, respectively while the unknown state of the bone is characterized by “?”; mt2 denotes both forward and reverse sequencing were successful using primers mt2-1 and mt2-2; 2-1 and 2-2 means that only forward or reverse sequencing was successful, respectively, 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 PCR.

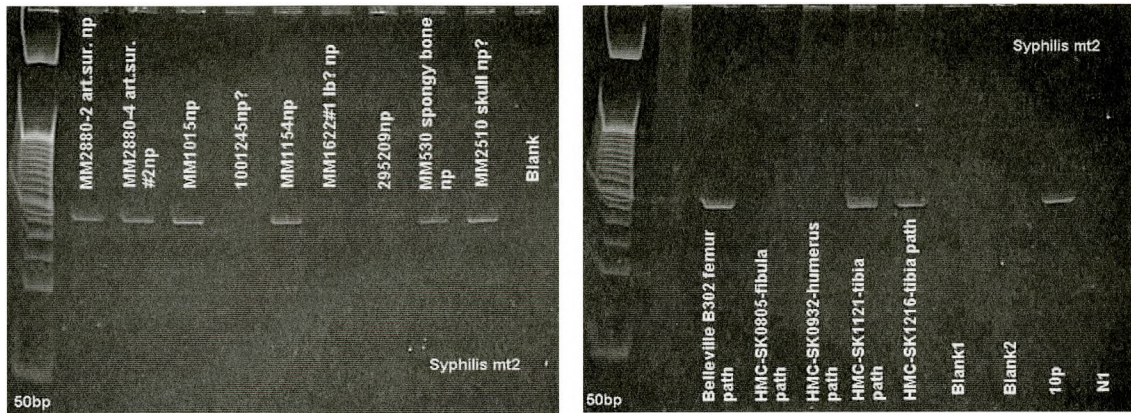


Figure 5.1: 8% non-denaturing polyacrylamide gels stained with ethidium bromide illustrating results for mtDNA for several samples. 10p is 10 pg of positive control DNA from K562 (Invitrogen). A 50 bp ladder was used.

Although the figure above illustrates that a positive result is not observed for samples Civil War 1001245 np?, 295209np, UK HMC94-SK805 fibula path and HMC94-SK932 humerus path, in subsequent amplification reactions using more $MgCl_2$ (to a maximum of 3mM), BSA (to a maximum of 2mg/ml) and AmpliTaq Gold polymerase (Perkin-Elmer) (to a maximum of 5U) were added to increase success rates or yields. Besides those listed above, supplementing the amplification reactions with these chemicals was also performed for Civil War MM1622 (no results were ever obtained for this individual), Civil War MM530 long bone and femur (no results were ever obtained for this sample), UK Whithorn (no results were ever obtained for this sample), UK Blackfriars SK77 and Belleville B302 radius. This shows that a number (almost one third) of the samples required the addition of chemicals to provide a positive result.

For those samples that did not amplify (Civil War MM1622, MM530 long bone and femur and UK Whithorn), an inhibition test was performed using the mtDNA protocol with the addition of positive human DNA (K562) at two different volumes (1 and 2.5 μ l, or 10 pg and 25 pg of DNA, respectively) (Figure 5.2) in conjunction with sample aDNA (to a maximum of 5 μ l) to the PCR reaction. Even at half the DNA concentration, PCR is still unsuccessful for UK Whithorn or Civil War MM1622. This test shows that there are some constituent(s) in these extractions that are inhibiting the PCR reactions.



Figure 5.2: 1.5% Agarose gel stained with SYBER® Green I dye illustrating the results of the inhibition test. Note: even with the addition of 2.5 μ l of modern human DNA (25 pg), negative results were still obtained for Whithorn, MM1622 long bone #2 and the bone dust.

Aligning the mtDNA sequences using BioEdit (Hall, 1999) reveals that matching DNA sequences for multiple tissue types of each individual are observed for UK HMC94-SK805, HMC94-SK932 (except for the humerus sample), HMC-SK1216 and Belleville B302 (see Table 5.3). Those individuals having a single tissue type, but matching forward and reverse sequences are Civil War MM1015 fibula, 1001245 rib, 295209 skull, MM2510 skull and UK Blackfriars SK77.

			16182	16183	16185	16189	16193	16212	16213	16223	16231	16234	16243	16255	16263	16264	16267	16270	16278	16292	16294	16296	16298	16301	16302	16304	16311	16313	16319	16324	16231	16266	16332	16344	16348	16390				
	Origin	Sample #	Bone Element																																					
1	<i>Civil War, USA</i>	1001949 MM2880-2	articular surface																																					
			bone dust (no decontamination)																																					
2		1001949 MM2880-4	articular surface						Y						R	Y																								
			bone dust (no decontamination)																																					
3		1002700 MM1015	fibula end																																					
4		1001245	rib					T	C		T						T																							
5		1002814 MM1154	skull?																																					
			skull																																					
			skull																																					
6		1000304 MM1622	long bone																																					
			long bone with lots of spongy																																					
			bone dust with foil particles																																					
7		1002422 MM530	long bone (femur?)																																					
			femur in foil																																					
			bone dust (no decontamination)																																					
8		295209	skull (shellac)																																					
9		1000873 MM2510	skull (black colouring)																																					
10	<i>Hull, England</i>	HMC-SK805	fibula																																					
			long bone																																					
11		HMC94-SK932	humerus																																					
			tooth																																					
			long bone																																					
12		HMC94-SK1121	fibula																																					
			right metatarsal																																					
			tibia																																					

Results show that when reverse sequencing is performed, it matches its predecessor in all occurrences (e.g., 295209). In several instances, not all of the DNA sequences from different tissue samples from the same individual match. For example, the humerus DNA sequence of individual UK HMC94-SK932 does not match the tooth and long bone samples; the right metatarsal DNA sequence of individual UK HMC94-SK1121 does not match the tibia and fibula. In other circumstances, comparing mtDNA sequences is rather difficult due to the possible presence of heteroplasmies and/or mixed bases at certain sites which could be the result of poor DNA preservation. For example, individual Civil War MM2880-4 possessed mixed bases at sites 16223 (T/C), 16255 (A/G), 16264 (T/C), 16298 (T/C), 16301 (C/T); Civil War MM1154 at sites 16187 (T/C), 16189 (T/C), 16223 (T/C), 16264 (T/C), 16270 (T/C), 16278 (C/T), 16302 (A/G?), 16311 (T/C), 16319 (A/G?); and Civil War MM2510 at sites 16185 (C/T), 16193 (C/T), 16332 (C/T), 16344 (C/T), 16348 (C/T).

When compared to the Cambridge Reference sequence (Anderson et al. 1981), Civil War 295209, UK HMC94-SK932 humerus, HMC94-SK1216, Blackfriars SK77 and Belleville B302 all match this reference (see Table 5.3). In fact, the majority of the samples tested using mtRadius (Fluxus Technology Ltd.) (Hall, 1999) all suggest European origins (see Table 5.4 below). 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.

Sample	Haplogroup	Country/Continent of Origin
MM2880-2	T	Europe
MM2880-4	H	Europe
MM1015	preV	Europe
1001245	L1b	Africa
MM1154	M/ambiguous*	Middle East
295209	H	Europe
MM2510	L2	Africa
HMC94-SK805	T	Europe
HMC94-SK932	U4	Europe
HMC94-SK1121	H	Europe
HMC94-SK1216	H	Europe
Blackfriars SK77	H	Europe
Belleville B302	H	Europe

Table 5.4: Haplogroups for those samples with positive mtDNA results using mtRadius (Fluxus Technology Ltd.) (Hall, 1999).

*M haplogroup was determined using ambiguous bases (e.g., N), but when these mixed bases were taken out of the calculation, an ambiguous haplogroup was found.

5.2.b Amelogenin:

When the amelogenin primers are used to sex the 16 individuals, only 10 (Table 2) could be amplified. Two of these, Civil War MM1015 and MM1154, are very tentative due to poor gel visualization (see Figure 5.3). This results in a 50% (8/16) success rate. As described in the Materials and Methods (see pages 66-68), morphological sex was assessed from/on the archaeological materials UK HMC94-SK805 (male), SK932 (male), SK1121 (female), SK1216 (male), Blackfriars SK77 (female) and Belleville B302 (male) and the historical records for the Civil War individuals stated that MM1015 is male, MM1154 is male, MM530 is female and 295209 is female. The molecular sex coincided with the morphological and historical records (in some instances, even with multiple amplifications [e.g., Civil War MM2510, UK HMC94-SK805, HMC94-SK932 and Belleville B302]) for all individuals where sex is

known except for Civil War MM530. In this case, the tentative molecular results conflict with the historical records. This particular sample consists of bone dust (which could not be decontaminated) from a long bone from MM530 and was collected by DY (a male). It is possible that this part of the sample was contaminated by the collector which was shown to be the case when the mtDNA was sequenced. Of the ten individuals whose sexual identity is known, the success rate of correct amelogenin sequence amplification results in a 50% (5/10) success rate. No molecular sexing results are obtained for Civil War 295209 or UK HMC94-SK1216.

It was noticed that not all samples from the same individual could be typed for sex. For example, only one skull fragment from Civil War MM1154; only the bone dust from Civil War MM1622 (although this could be due to contamination by the sampler); only the long bone from Civil War MM530; only the long bone fragment from UK HMC94-SK805; and only the tibia from UK HMC94-SK1121 could be typed for sex using the amelogenin primers.

In several instances only one band at 119 bps is visualized on the gel when two should have been present to denote a male molecular sex type. This occurs for Civil War MM1015 fibula, MM1154 skull, MM530 long bone, MM2510 skull and Belleville B302 radius (see Figure 5.3). This can be explained by X chromosome allelic drop out which occurs when little DNA is preserved and competition results with the shorter amplicon (from the Y chromosome) amplifying more readily. Due to the design of this protocol, X chromosome drop out for males does not lead to incorrect sexing as the Y chromosome amplicon is preferentially selected for amplification.

Of interest to note is that the humerus of UK HMC94-SK932 is sex typed as female, while both the tooth and long bone fragment from this individual are male (remember the humerus mtDNA was different for this individual too) (Figure 5.3c and d). Another possibility for this anomaly could be related to contamination by the researcher (who is female), although this should have resulted in a much brighter band as modern DNA is much better preserved than aDNA.

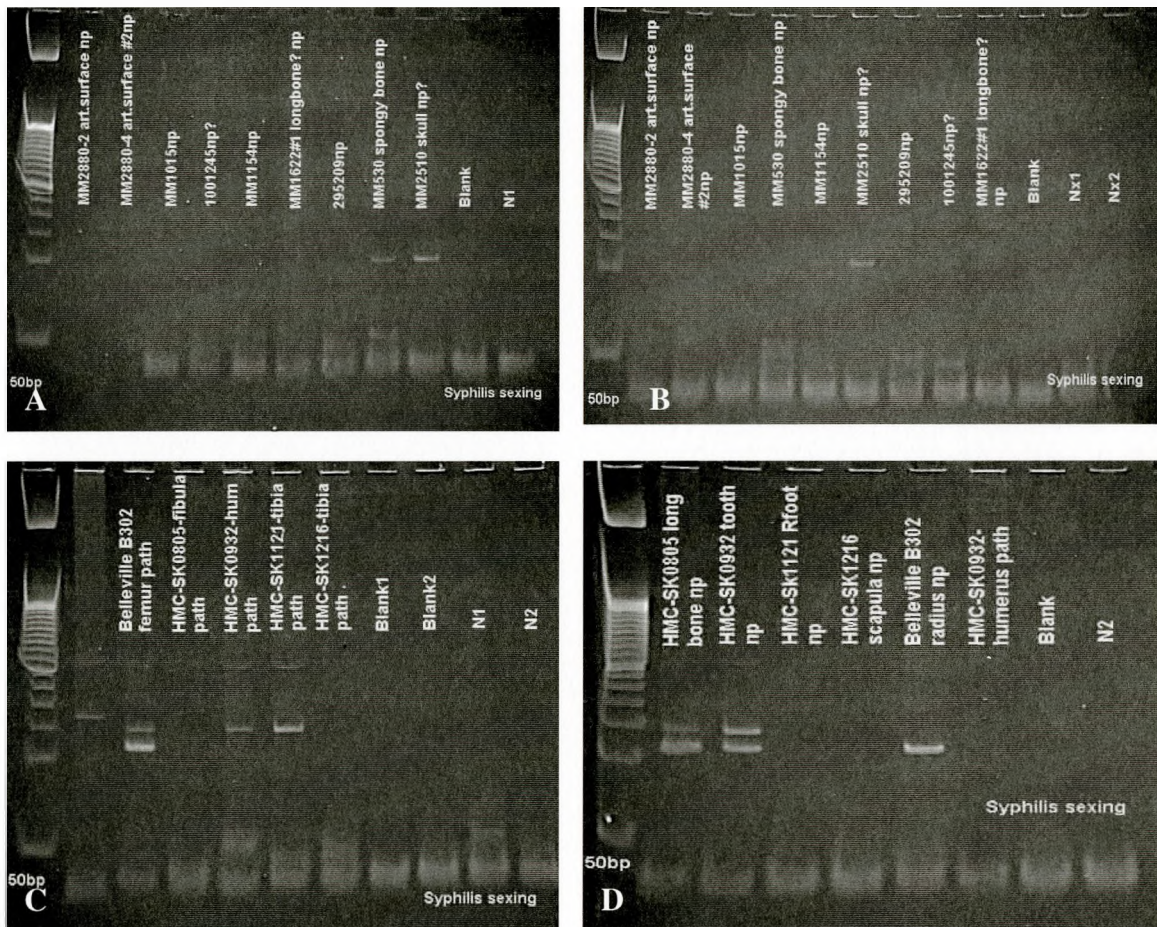


Figure 5.3 (A, B, C, D): 8% non-denaturing polyacrylamide gels stained with ethidium bromide illustrating results for amelogenin sex typing. One band at 154 bp denotes the X chromosome amplicon and another band at 119 bps represents the Y chromosome amplicon. Note the allelic drop out for the X chromosome in MM530, MM2510 and Belleville B302 radius np. Note the differences in sex typing for HMC94-SK932. A 50 bp ladder was used for control.

5.2.c *Treponema pallidum* subsp. *pallidum*

In the end, four different areas of the *Treponema* genome were analyzed for these 16 samples. These consisted of two different protocols for the 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 five of these different protocols provide no positive results for all 16 human samples in this project (to illustrate, please see Figures 5.4-5.6).

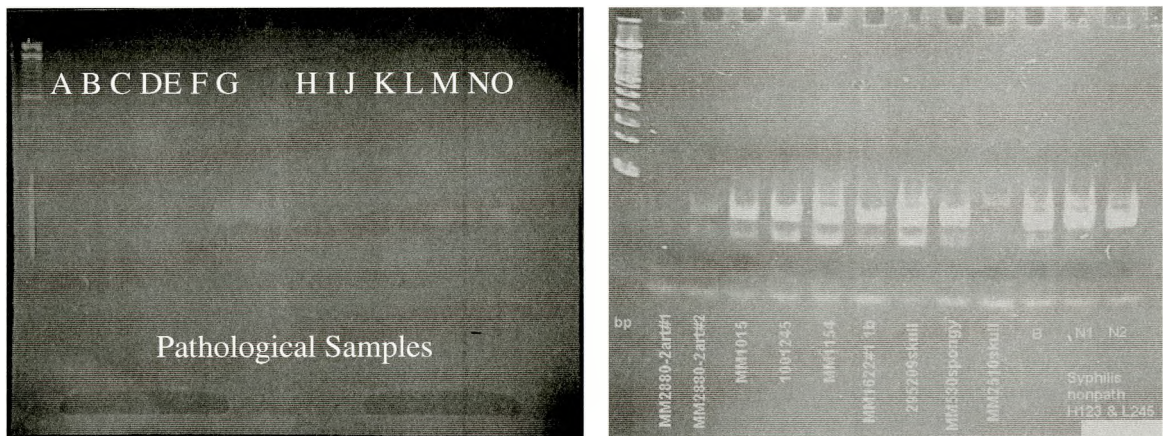


Figure 5.4: 4% agarose gel (on the left) and 8% non-denaturing polyacrylamide gel (on the right) stained with ethidium bromide illustrating negative results for the same samples using the Kolman et al. (1999) method. A. Belleville B302 femur, B. HMC94-SK805, C: HMC94-SK932, D: HMC94-SK1121, E: HMC94-SK1216, F: Blank 1, G: Blank 2, H. SK77, I. Whithorn gumma, J. HMC94-SK932, K. HMC94-SK1121, L. HMC94-SK1216, M. Blank 1, N. Negative 1, and O: Negative 2. The estimated amplicon size is 120 bps. 100 bp ladder was used for control.

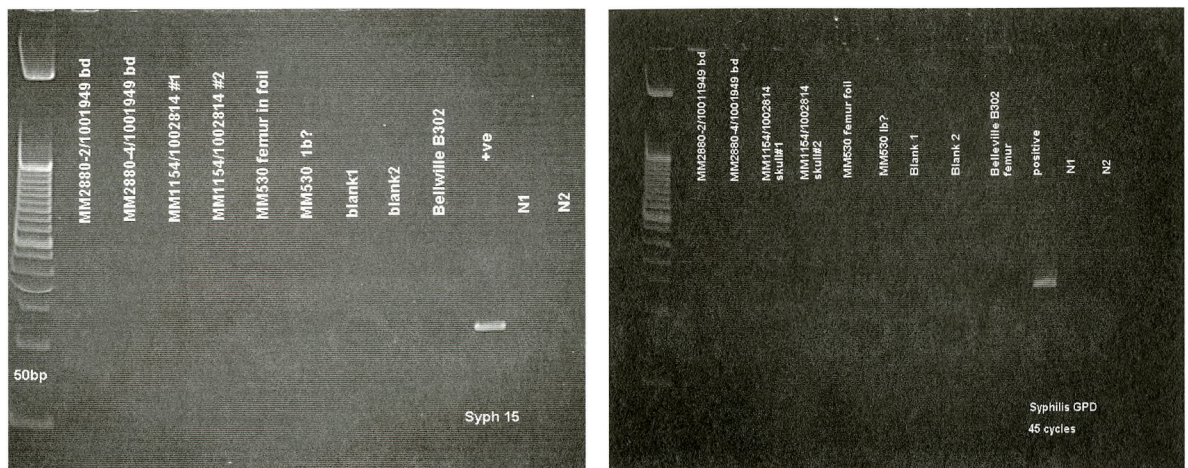


Figure 5.5: 8% non-denaturing polyacrylamide gels stained with ethidium bromide illustrating negative results for 15-kDa (on the left) and GPD ARMS (on the right) tests. The Positive (+ve) consists of the cloned material for each of these genes (estimated amplicon sizes are 127 bps and 139 bps, respectively). The estimated amplicon sizes for these two genes are 147 bps and 169 bps, respectively 50 bp ladder was used for control.

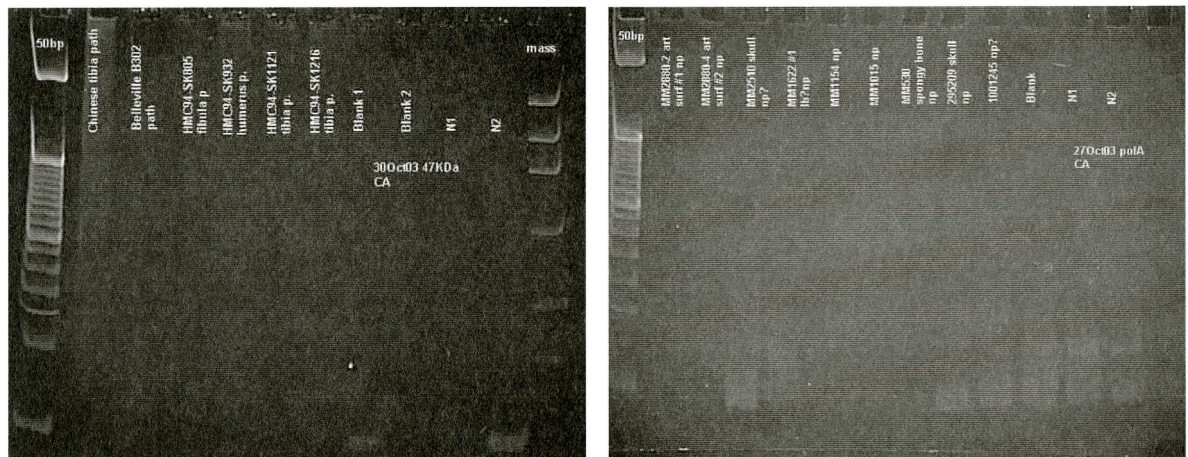


Figure 5.6: 8% non-denaturing polyacrylamide gels stained with ethidium bromide illustrating negative results for the 47-kDa membrane protein (on the left) and the DNA Polymerase I (on the right) genes. The estimated amplicon sizes are 196 bps and 377 bps, respectively 50 bp ladder was used for control.

5.2.1 Rabbit Testing

Different tissue types were sampled from two adult New Zealand white male rabbits which were in different stages of syphilis. Twelve samples were taken from

“Rabbit 12” and three were taken from “Rabbit C” (see Table 5.5 below). All samples were subjected to the five different PCR protocols that identify treponemal bacteria (for results please see Table 5.5).

Positive Test Results						
Rabbit	Sample Taken	Kolman et al. (1999)	15-kDa ARMS	GPD ARMS	47-kDa	<i>polA</i>
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			*			*

Table 5.5: Samples taken from adult New Zealand white male rabbits injected with *Treponema pallidum* subsp. *pallidum* and the results of the molecular tests which identify this bacteria. * = positive result; ☒ = faint bands on gel

More positive results are obtained from the rabbit with the acute form of syphilis (“Rabbit C”) rather than the rabbit with a prolonged syphilis infection (“Rabbit 12”) (See Figures 5.7 and 5.8).

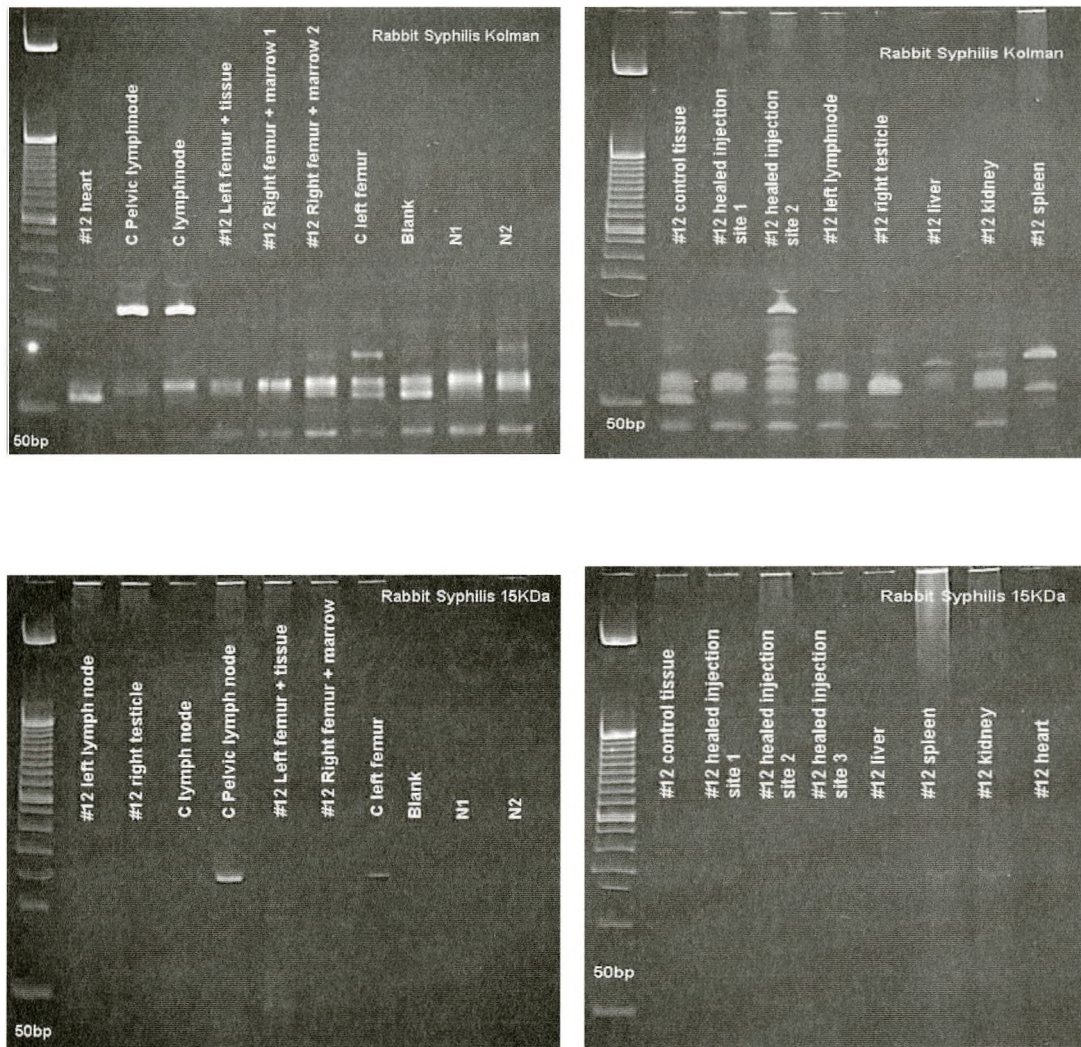


Figure 5.7: 8% non-denaturing polyacrylamide gels stained with ethidium bromide illustrating positive results for the 15-kDa lipoprotein genes using the Kolman et al. (1999) protocol (upper gels) and the ARMS protocol (lower gels) on modern rabbit tissues. The estimated amplicon sizes for these two protocols are 120 bps and 147 bps, respectively. 50 bp ladder was used for control.

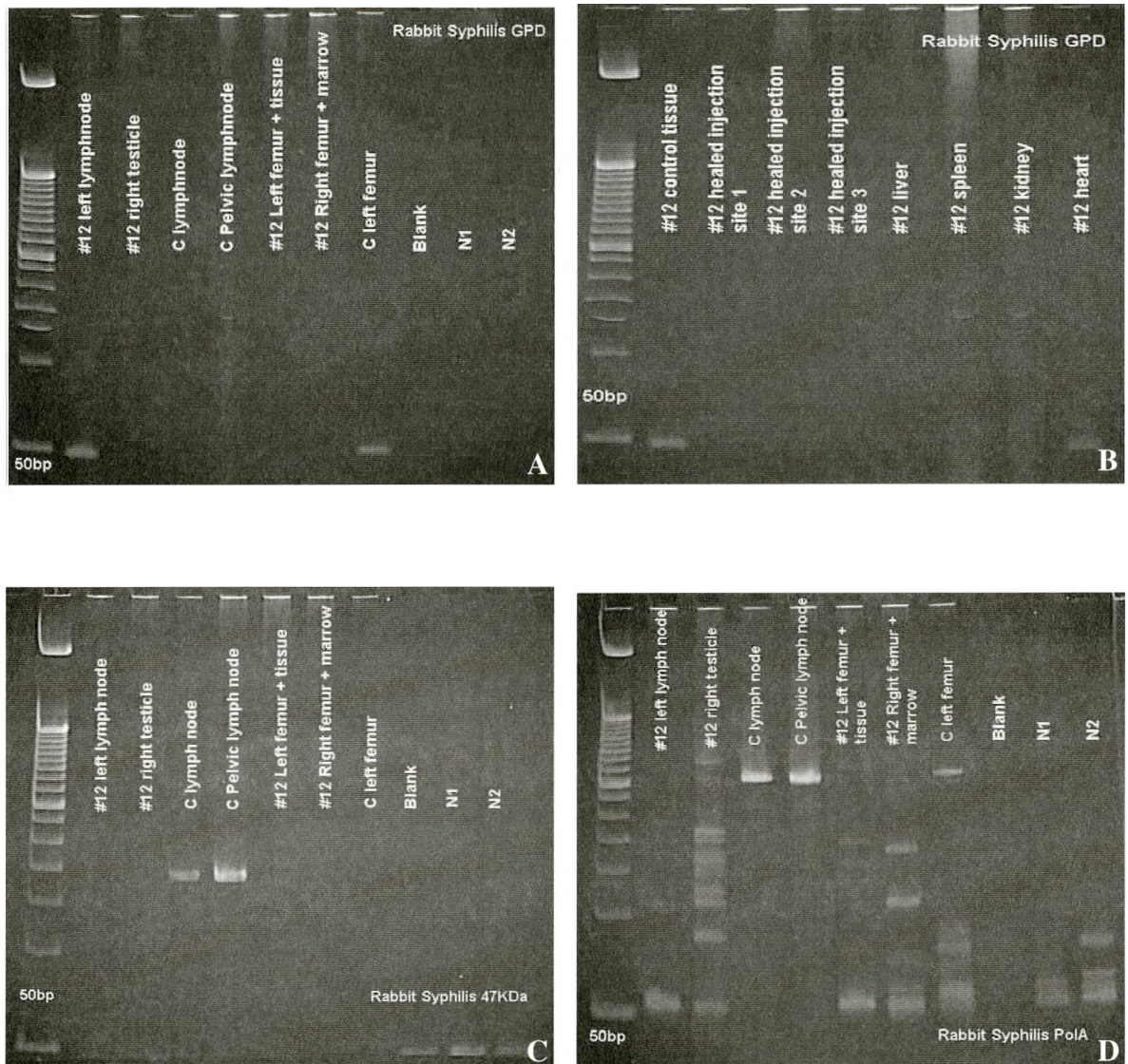


Figure 5.8: 8% non-denaturing polyacrylamide gels stained with ethidium bromide illustrating positive results for the GPD (upper gels, A and B), the 47-kDa membrane protein (gel C) and the Polymerase I (gel D) genes on modern rabbit tissues. The estimated amplicon sizes for these protocols are 169 bps, 196 bps and 377 bps, respectively 50 bp ladder was used for control.

These results also show that the 15-kDa ARMS and *poIA* protocols are more sensitive than the others. Several of these positive results were sequenced to make sure the right DNA amplicon was amplified/targeted. These results coincide with the GenBank sequences (see Figures 5.9 and 5.10) except near the beginning/end of the sequence run (approximately the first 20bps) which is a normal observation.

	185 195 205 215 225 235
15kDa lipo	GTGCTGAGCA GGATGTCTCT ATGAGTTATA AAGAGTTTTC GACGCGTGCG GCGCGGCGTT
Endemicum
Pertenuae
CFEMUR
12INJECT2	-----NNTC. CTACNCCG.G ...T.ACGC .GA.NNNAC.

	245 255 265 275 285 295
15kDa lipo	CTGCCCTTTT GACGTTGCGT GTTCTTGAGC GTCTCAGCGC TTTACGCACA TCGGTAGTGG
EndemicumT.....
PertenuaeT.....
CFEMUR	----NCNCN N.T.....
12INJECT2

	305 315 325 335 345 355
15kDa lipo	CTTCTCTTTT CCGATGGTC GTGGTGTAAT GGTCGGGAGT AAGACAGTGG GCTGAGCAGT
Endemicum
Pertenuae
CFEMUR
12INJECT2ANNNN.....

	365 375 385 395 405 415
15kDa lipo	CGGGCGATGC TGTGGGGGGC TTGTGTAGGT GTATACCCAG TTGTATACT GTGGCACTCG
EndemicumC.....
PertenuaeC.....
CFEMUR
12INJECT2

Figure 5.9: Sequence alignment of *T.p.* subsp. *pallidum* 15-kDa lipoprotein gene using GenBank TPU75465. Endemicum (GenBank TPU75464) and Pertenuae (GenBank TPU75463) are DNA sequences for endemic syphilis and yaws, respectively. CFEMUR is the bone sample taken from Rabbit C which was (amplified and) sequenced using primer TPALCLIPO from the ARMS test. 12INJECT2 is the skin sample taken from Rabbit 12 injection site 2 which was (amplified and) sequenced using primer L243 from Kolman et al. (1999).

	1745	1755	1765	1775	1785	1795
Pola	ACTTAAACGT	ATTATTGATT	GCGCGTGTGC	GAATGGTGTG	GTCGCGTTTG	ATTGTGAAAC
CPLYMPH						NC..N.....
CFEMUR			N.CTG	CCG.C..CN.	NA..GTC...	T..N.AGNNA

	1805	1815	1825	1835	1845	1855
Pola	GGATGGATTG	CATCCGCACG	ATACACGT__	_CTGGTCGGA	TTTTCGATCT	GCTTTCAGGA
CPLYMPH						
CFEMUR	CC.CTCG.C.	GCGN...TTA	T...N.AGTC	A.....		

	1865	1875	1885	1895	1905	1915
Pola	AGCAGAGGCT	TTTATGTTT	CTCTTATTGT	TCCGGACGTT	TCTCTTCATA	CCGAGTCAAC
CPLYMPH						
CFEMUR						

	1925	1935	1945	1955	1965	1975
Pola	TCAGTGTA	TGTGCACGTA	GCACTAATGT	CGAGACTGAA	AAGGAGTGCA	CAGAACAGCA
CPLYMPH						
CFEMUR						

	1985	1995	2005	2015	2025	2035
Pola	TGGGGTATCT	GCATCTGCTG	TGCAGGATCC	GGCATATGTC	CAAGCTGTCA	TGCACCAGCT
CPLYMPH						
CFEMUR						

	2045	2055	2065	2075	2085	2095
Pola	TCGACGTCTT	TGGAATGATG	AGACGCTCAC	ACTTGTTATG	CATAATGGAA	AGTTTGATTA
CPLYMPH						
CFEMUR						

	2105	2115	2125	2135	2145	
Pola	TCACGTTATG	CATCGTGCAG	GCGTTTTTGA	GCACTGTGCA	TGTAATATTT	T
CPLYMPH				...ACTN.TG	ANNNNNNNNN	N
CFEMUR			AN	NNN	

Figure 5.10: Sequence alignment of *T.p.* subsp. *pallidum* Polymerase I gene using GenBank TPU57757. CPLYMPH is the pelvic lymph node sample and CFEMUR is the bone sample taken from Rabbit C. Both of these samples were (amplified and) sequenced using primer F1 (forward primer) from Liu et al. (2001).

Another important observation is the amplification of treponemal DNA from the femur of Rabbit C. This is the first time bone has been used in PCR techniques centered

on the identification of syphilitic DNA from a positive clinical sample. 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).

CHAPTER SIX:

DISCUSSION

Multiple lines of evidence in support of an hypothesis are fundamental in physical anthropology, especially paleopathology. This allows for an improved diagnosis and a better understanding of the disease process. This study has relied on macroscopic (based on observations recorded in historical documents and by personal observations), microscopic and molecular approaches to aid in the identification of syphilis in historic skeletonized individuals from both North America and the United Kingdom. In most circumstances, syphilitic infection in these individuals could be diagnosed using macroscopic and/or microscopic methods. Although for the majority of the samples the use of molecular protocols (i.e., mtDNA and sometimes nuclear DNA) were successful, isolation of treponemal bacteria specific to *T.p.* subsp. *pallidum* was not. Investigating the reasons why this has occurred for this particular project will lead one in many directions: alternative diagnoses, diagenesis, preservation of DNA in general, preservation of treponemal organisms and their DNA specifically, and bone as an inappropriate environment for treponemal organisms in the tertiary stage of the disease.

6.1 Differential Diagnoses

To begin, it must be recognized that when other infectious diseases (e.g., TB) are ruled out in the differential diagnoses of the individuals in this study, pathogenic treponemal diseases (i.e., yaws and endemic syphilis) may still be an alternative. However, when climate and geography (latitude and rural vs. urban environment) are

considered, it is more likely that these individuals suffered from venereal syphilis rather than other treponematoses.

Several of the Civil War samples (i.e., MM2880-2, MM2880-4, 1001245, MM1622, 295209 and MM2510) used in this project were purchased by the Armed Forces Institute of Pathology (also known as the American Medical Museum) from personal collections in the 1800s. No autopsy reports involving full body examination were performed for these individuals and as a result, the diagnoses are based on those provided by museum curators (assumably from the bone elements obtained) and the original collectors. Diagnoses were performed initially by the curator, P. Sledzik, and then confirmed by one of the samplers (SR Saunders) and from pictures of the bones themselves if photos were taken. It is also problematic that the whole skeleton was not available for lesion distribution analysis as this can aid in the diagnosis. These circumstances have led to less confident interpretations of the expression of venereal syphilis in these six individuals.

The ‘enlarged and thickened periostitis’ of the tibiae documented in MM2880-2 and MM2880-4 may have been caused by a number of non-specific infections related to trauma, hematogenous osteomyelitis, etc. However, excess osseous production along the diaphysis of the tibia is quite characteristic of syphilis (Aufderheide and Rodríguez-Martín, 1998; Ortner and Putschar, 1985) and should not be disregarded. Since both tibiae are infected, cancerous states and hematogenous osteomyelitis can be ruled out. In addition, because there is an excess growth of bone, tuberculosis is not accepted as it

normally involves destructive lesions. Unfortunately there is no mention of “saber shin” formation in the documents which would have been most helpful in this respect.

Individual 1001245 may have been suffering from tuberculosis as lytic/erosive lesions of the thoracic vertebrae were observed in the photograph of this specimen (see Plate 4.2) which is indicative of this disease (Aufderheide and Rodríguez-Martín, 1998; Ortner and Putschar, 1985). This is quite unlike syphilis which normally alters either the cervical or lumbar regions with *both* osteoclastic and osteoblastic reactions. These observations suggest that tuberculosis is a more likely candidate than syphilis, however, the latter cannot be ruled out definitively (Kampmeier notes erosion of the spine and ribs by aortic aneurysm in late syphilis [1964]).

Descriptions written in 1883 and personal observations of the cranium (ulcerative and non-ulcerative lesions), femora (cloacae and proliferation of bone) and tibiae (lytic and remodeled areas) of individual MM1622 all suggest syphilitic action. This is based on the fact that multiple areas of the skeleton are affected by both destructive and proliferative osseous responses to the presence of disease. As a result, tuberculosis, cancerous states and hematogenous osteomyelitis can be excluded.

Erosion of the palate of individual 295209 is an indicative feature of syphilis, but is also observed in leprosy. However, leprosy cases do not involve erosion of the frontal bone which has occurred in this individual. Complete destruction of the calvarial inner table in this particular case, although rare, can occur in the final stages of syphilis (Aufderheide and Rodríguez-Martín, 1998). It must also be pointed out that when observing the picture of the skull (see Plate 4.6), the destruction of the bone has occurred

from the outer table inwards (note direction of beveling at the extremities of lesion) and thus, tuberculosis can be excluded (usually destruction occurs from the inner table to the outer table).

Areas of ulceration found on the parietals and temporal bone of individual MM2510 are relatively diagnostic of syphilis, but can also be caused by other diseases. Metastatic carcinoma and multiple myeloma can both affect the skull, but the lesions of metastatic carcinoma are generally expansile and not necrotic while multiple myeloma is purely lytic and smaller in comparison to syphilis.

The remaining Civil War specimens (i.e., 1002700, MM1154, and MM530) have relatively detailed autopsy and/or medical reports which provide information more conducive to the diagnosis of venereal syphilis. The combination of tibia and fibula periostitis and osteomyelitis with calvarial erosive lesions that were not associated with any tumors or abscesses for individual 1002700 as well as hemiplegia/paralysis (signs of tertiary syphilis) identified shortly before his death, point strongly to venereal syphilis. The calvarium of individual MM1154 is quite thickened which is often seen in Paget's disease. However, the presence of nodes, cavitations and stellate scarring is more indicative of venereal syphilis. Finally, records for individual MM530 appear to be somewhat contradictory in that the autopsy report is clearly describing what seem to be alterations caused by tuberculosis infection (i.e., pleuritic lesions and tubercles of the lung as well as amyloid degeneration of several organs), but the remaining documents specifically use the term "syphilitic" when describing some of the bone lesions. One must remember that tubercles are caused by granulomatous inflammation which can also

be found in syphilitic cases (in the form of gummata instead of tubercles) (Cotran et al., 1999). The chronic inflammation of the periosteum and cortical bone in conjunction with more proliferative rather than erosive lesions of the tibiae and femora, point more to a syphilitic causation as well. It is possible that this particular individual may have suffered from both infectious diseases as one can exacerbate the other.

The diagnosis of syphilis in the remaining samples was based either upon previous reports (Whithorn [Cardy et al., 1997], Blackfriars SK77 [Roberts, 1994] and Belleville [Jimenez, 1991]) or personal observation of the material (HMC94 individuals). Since caries sicca is a distinctive indicator of treponemal infection (Hackett, 1975), no differential diagnosis is needed for Whithorn, Blackfriars SK77, Belleville B302, HMC94-SK932 and HMC94-SK1216. Also, caries sicca in combination with the involvement of multiple bones in the postcranial skeleton in the form of both proliferative and destructive lesions lends more to the diagnosis of syphilis than any other disease.

Diffuse response to infection, which may be of a nonspecific nature, is noted by the presence of periostitis and osteitis for all upper and lower long bones of individual HMC94-SK805. This individual also presented with several ankylosed thoracic vertebrae which may be diagnosed as diffuse idiopathic skeletal hyperostosis (DISH) due to its formation (looks like dripping candle wax – smooth calcification of bone), placement (anteriorly) and age of the individual (Aufderheide and Rodríguez-Martín, 1998). The presence of this manifestation still does not exclude syphilis infection in other regions of this individual as its cause is unknown. Slight involvement of metatarsals may suggest

leprosy, but since the lesions in this area of the skeleton are more proliferative (periostitis), leprosy can be ruled out as it usually causes more destruction.

Individual HMC94-SK1121 presents with cribra orbitalia which may be caused by a frank nutritional deficiency and/or infection. Infection is prevalent in this individual as all upper and lower long bones are affected by periostitis and/or osteitis to such an extent that in some cases the topography of the bone is completely altered. These characteristics help to rule out tuberculosis (normally destructive) and hematogenous osteomyelitis (normally involves one bone), and are more characteristic of syphilis.

6.2 Histological Identification of Syphilis

Differential diagnoses are more complete with the addition of microscopic analysis. When this additional approach was applied to a few of the specimens, their micro-architecture showed the presence of both proliferative and osteolytic processes in the form of periostitis, osteitis (compact bone involved) and/or osteomyelitis (medullary cavity involved) which are indicative of inflammatory development. Although Polsters and Grenzstreifen are also supposedly characteristic of leprosy and hematogenous osteomyelitis, they vary slightly in morphology (i.e., comparisons between these diseases show that Polsters are “rudimentarily developed and relatively flat” and periosteal irregular formation, respectively and Grenzstreifen are fragmentary or not present, respectively) from those observed in treponemal infections (Schultz, 2001:126; Schultz, 2003 and Schultz and Roberts, 2001). These two structures identified in HMC94-SK805, SK1121 and Belleville B302 point more to a treponemal infection, rather than a leprosy

infection or hematogenous osteomyelitis because they are relatively large, distinct entities with organized structures.

This study has revealed that the paleohistopathological structures suggested by Schultz (1994, 2001 and 2003), are only rough guidelines in identifying syphilis as their size and shape appear to be highly variable. I feel that the structures observed in this study (i.e., Polsters and Grenzstreifen) are indeed those described and illustrated by Schultz, but that their morphological appearance is slightly different. In his work, Schultz fails to truly explain or illustrate the range of histomorphological variability and development*. For example when discussing Grenzstreifen, Schultz (1994:65) explains that he has seen cases where only fragments or residues of this structure exist when inflammatory processes are more completely healed. However, no more explanation, nor an illustration, is provided for the reader to act as a comparison or guide for this stage of infection. He also states that the slow progression of Polster formation common to treponemal disease displays a well-defined and regular pattern of “pillow-like newly built bone” (Schultz, 2001:126) that can be ‘sometimes’ separated from each other by “small crypt-like gaps” (Schultz, 1994:65). In this study both HMC94-SK1121 fibula and tibia presented with this structure, but when comparing their morphology some differences were noticed. The fibula specimen had clear, well-separated (i.e., relatively large crevasses separating the structures) and distinct extensions, while the tibial formations were more bulbous, and barely distinct (i.e., little space separated the structures) (compare Plate 5.28 and 5.32). Comparison of Polsters within the same region of a

* This is not only a problem with paleohistopathology, but also paleopathology in general.

histological section revealed that they varied in size and shape (see Plate 5.28). This variability was also observed when looking at Schultz's three major publications on identifying disease in the past via histology (especially pertaining to the identification of syphilis), but it is never discussed (e.g., compare Figure 2 with B [1994] and with Figure 8B [2001]).

One is left to question how variable these Polster formations can be when they no longer resemble or can be considered of treponemal origin. It is possible that at the time of death, very little bone proliferation had occurred to provide a full-fledged well-defined pillow-like structure and instead, all the microscopist can observe are undulations along the periosteal surface. For example, very small extensions along the new periosteal surface were observed in HMC94-SK805 fibula (see Plates 5.6, 5.16 and 5.25) and Belleville B302 femur (Plate 5.27). These manifestations could be the beginning stages of Polster formation. However on the other hand, very large Polster-like formations were noted in the macroscopic views of HMC94-SK1216 tibia fragment 1 and 2 (e.g., Plates A.7 and A.8 in the Appendix), but poor preservation did not allow for the observation of defined lamellar bone within these structures.

It was also noticed throughout this study that Polsters (and Grenzstreifen) were only found in focal areas. Although Schultz makes no mention of this, it is assumed that such formations will only occur in areas of observable (macroscopic) lesions. However, if an entire circumference of a long bone shaft is visibly altered, but only a few Polsters (or Grenzstreifen) are identified, what does this say about the disease process or the criteria used to identify it microscopically? This becomes a valuable question when one

is faced with only fragments of bone to help in differential diagnoses (i.e., perhaps the fragment that is preserved does not exhibit these manifestations, but had the adjacent area been preserved they could have been present). This may have been the case for individuals HMC94-SK932 and SK1216 (Plate 5.37).

On the other end of the spectrum when the disease process has been prolonged, one must question how these structures are manifested or how they are altered since bone is not a static entity. For example, it was noticed that some of the Polster formations in HMC94-SK1121 fibula and tibia contained Haversian canals (Plates 5.28-5.30). In this same individual, several Polsters were enveloped by new bone proliferation (e.g., see Plate 5.32) making their identification difficult as they were no longer distinct extensions of the periosteal surface. Generally, the presence of osteons and osteoblastic activity points to a sufficient elapse of time allowing for remodeling. The presence of osteons is only very briefly introduced by Schultz when he states, “[i]t seems that, in the cases of syphilitic inflammation, bone remodeling could also produce newly built Haversian systems. However, this could only be possible, if active disease is no longer in progress” (1994:65). In a later publication he states that the microscopist will see “vestiges of an extensive remodeling process” (Schultz 2001:128) as the disease progresses, but does not provide the reader with examples of such occurrences. As a result, most of Schultz’s criteria are based on *active* forms of treponemal disease which are not indicative of the range (i.e., from active, latent to healed) paleopathologists and/or paleohistopathologists come across when observing the archaeological record. Since syphilis is such a

prolonged disease with many stages, it seems imperative that a discussion of chronic, prolonged exposure to inflammation needs to be addressed.

As pointed out earlier (also in Chapter 3: Identification of Syphilis), Polsters are not just indicative of syphilis, but can also be found in endemic syphilis, leprosy and hematogenous osteomyelitis (Schultz, 2001 and 2003). Schultz (2001:126) contrasts the latter two with treponemal infection by their slight difference in morphology. It is this author's belief that any disease which entails episodes of inflammation or infection and is of a chronic relapsing nature, will present with similar bony reactions, especially when an increase in vascularization occurs. This is identified by Schultz (2001) when he acknowledges that endemic syphilis also manifests Polsters and Grenzstreifen. However, it is also believed that further, more extensive histological research will reveal that yaws also presents with these formations.

6.3 Diagenesis and its Relationship to Histology and Molecular Preservation

Diagenesis of bone material results in any kind of alteration or change from the original morphology of the osseous matrix. This process can include dissolution, precipitation, adsorption, mineral replacement, and recrystallization which are dependent on the physical and chemical characteristics of the burial environment as well as intrinsic factors (e.g., Elliot and Grime, 1993; Grupe and Garland, 1993; Grupe and Piepenbrink, 1989; Grupe et al., 1993; Hedges and Millard, 1995; Stout, 1978; Von Endt and Ortner, 1984; White and Hannus, 1993). Researchers must be aware of these processes in order to understand why the histomorphological, chemical and molecular nature of bone has been altered.

Microscopic views of those bones that could be sampled (MM530 femur, HMC94-SK805 fibula, SK932 humerus and long bone, SK1121 fibula and tibia, SK1216 tibia, Blackfriars SK77 rib and Belleville B302 femur) all show signs to some extent of poor preservation, and hence diagenesis, of histological structures. For those samples that had little to no birefringence (i.e., preservation of little to no mineralized collagen), two possibilities exist to explain this occurrence. Osseous structures can be altered when bone collagen is destroyed via microorganism invasion leading to demineralization (Hackett, 1981) and by hydrolysis of chemical bonds found in collagen (Henderson 1987). Microorganism invasion was indicated in several of the bone samples (e.g., HMC94-SK932 and SK1121) by the presence of Wedl canals. Their observation was based on the orientation of the destruction (perpendicular to or not in line with lamellae), their characteristic shape (uniform size, irregular outline/walls, branching) and absence of mineral redeposition (lack of hypermineralization) (following Hackett, 1981). Collagen preservation was ascertained inadvertently for this study via C/N ratios performed during C14-dating of the HMC94 bone samples (except for HMC94-SK805) (Tom Higham, personal communication). These ratios average around 3.2 coinciding with a rough relative estimate of good collagen preservation (Schwarcz and Schoeninger, 1991) within these bones.

In general, those samples with poor micro-architecture also exhibited poor molecular results. For example, no molecular results could be obtained, even at the mtDNA level, for MM530 which had no birefringence or osteon structures preserved. This correlation does not seem to fit with Blackfriars SK77 as little preservation of

histological structures were observed, but mtDNA and nuclear DNA could be amplified from this sample. On the contrary, strong molecular results (i.e., success at the nuclear level) were observed for HMC94-SK805 and Belleville B302 which had well-preserved micro-architecture. Mixed histological preservation (i.e., areas of no birefringence, presence of Wedl canals and/or fungal inclusions) did present with successful mtDNA and nuclear DNA results (e.g., HMC94-SK932 and SK1121). HMC94-SK1216 did have mixed histological preservation as well, but it did not provide nuclear DNA results. This could be due to the fact that its osseous structures were more poorly preserved than those observed in HMC94-SK932 and SK1121.

Correlation between histological preservation and DNA success has been identified by other researchers (Barnes et al., 2000; Cipollaro et al., 1998 and 1999; Colson et al., 1997; Hagelberg et al., 1991; Haynes et al., 2002; Savoré, 2000), but negated by others (e.g., Mays et al., 2001). This correlation has been related to the increase in porosity that occurs with diagenesis which allows for more groundwater to percolate and react with bone constituents (e.g., hydrolysis of DNA molecules) (Hedges and Millard, 1995; Von Endt and Ortner, 1984). Although not discussed in the literature, microorganism focal destruction may lead to degradation of DNA molecules as the microorganisms destroy bone and the mineral portion (which DNA binds to). It will be interesting to see if future studies find a relationship between DNA preservation and the amount of microorganism destruction in bone.

All histological sections from individuals of the HMC94 site showed red/brown staining. Upon further investigation, the archaeologist for the site, David Evans, recounts

that the specimens used in this project came from shroud burials with some surviving textiles that should have been off-white or grey-white in colour, but when excavated were stained red. This difference in colour has been attributed to reactions which had occurred between the tannins in the vegetable dyes used in the over garments and the prevailing soil conditions (alluvial warp clays) (David Evans, personal communication). It was also noticed during the excavation that a similar red colour left behind in a silhouette of a body was observed on the basal oak planks of one of the coffins due to the contact of the clothes with the wood.

Without any chemical and mineral tests of the soil, it can also be hypothesized that this staining could have been caused by iron in the soil. According to Schultz (2001:117-118), iron can be a bone preserving element as he noticed better microscopic preservation when bone had been in contact with high concentrations of iron in the soil and in particular, when in close contact with an iron sword. It is of interest to note that those samples with the most prominent forms of staining, HMC94-SK805 and SK1121, had the best histomorphological preservation which gradually decreased as one moved to the endosteal surface of the bone. This clinal variation reveals a distinct effect of the staining and the preservation of bone micro-architecture within these samples. Iron is a metal that can contribute to oxidation reactions and as such, its presence may indicate an oxidizing environment. Unfortunately, because of iron's oxidizing capabilities it can attack the 3-4 carbon bond of the deoxyribose in the DNA molecule, causing ring fragmentation and strand scission (Poinar, 1997) which will ultimately inhibit the PCR reaction (Poinar, 2002). Oxidation reactions can also affect the nitrogenous bases

(Poinar, 1997). Such alterations to the DNA molecule suggest that the HMC94 samples should have had major problems during PCR, however this does not seem to be the case. Except for HMC94-SK1216, the majority of the samples from this site could be sex-typed using the nuclear genome, suggesting the DNA was relatively well preserved. As a result, it is suggested that the staining was not from an iron-containing constituent, but perhaps another chemical.

The HMC94 site was also waterlogged from about one metre below the surviving ground level producing an anoxic or anaerobic environment (David Evans, personal communication). This has led to excellent preservation of organic remains (e.g., bone, textiles, wood) identified during the excavation at the macroscopic level, as well as the microscopic and molecular levels identified in this project. Even though water is considered detrimental to DNA preservation, it will not be as harmful when it provides a *stable* environment (i.e., when no flow of constituents between bone and water occurs). This helps to create an equilibrium between bone and soil/water constituents and thus a decrease in degradation of bone mineral or hydroxyapatite. Several studies have proven that when groundwater was continuously present, the activity of organisms responsible for histological destruction was limited (Hanson and Buikstra, 1987; Millard and Hedges, 1995).

One area of taphonomy that is not well studied when it comes to pathological lesions in skeletons is how its process of degradation and diagenesis may differ from non-pathological bones. By observation alone, lesions have increased surface areas due to proliferation of bone and tend to be more porous due to osteoclastic actions as a result of

the inflammatory process. In fact, it has been shown that a positive correlation exists between decay and porosity of bone (Nicholson, 1996). When placed in conjunction with microorganism invasion, the porosity of bone is increased even more (also suggested by Elliot and Grime, 1993). These attributes allow for an increased interaction (e.g., dissolution, precipitation, adsorption, etc) with the burial environment via water movement (Hedges and Millard, 1995) and hence, more opportunities for both organic and inorganic alterations/destruction. With an increase in groundwater diffusion comes an increase in hydrolysis of the protein-mineral bond (Garland, 1987) and hence, less preservation of intact DNA molecules. This is related to recrystallization of HAP into different conformations which disallow the re-binding of DNA. Bell's work (1990) in the area of taphonomy and pathology also suggests that differences in lamellar arrangement of bone, distribution of external and internal surfaces and degree of mineralization often observed in pathologically altered bone plays an important role in the progression of diagenesis itself. For example, Bell (1990) noted a higher degree of diagenetic alteration to the periosteal aspect of specimens which had periostitis and an infilled medullary cavity compared to those specimens with open medullary cavities (more alterations were noted here for both non and pathological bones).

Intrinsic qualities of bodies are also considered by some to be more important in the initial stages of decomposition than the soil in which it is buried (Janaway, 1985). This is related to the body's ability to create its own micro-environment with endogenous micro-flora already present within. This is also supported by evidence in forensic medicine which has shown that the presence of antemortem injury or infection may

increase the rate of decomposition due to larger areas being exposed for bacterial decomposition (Rentoul and Smith, 1973). As autolysis is temperature dependent and the rate is increased at higher temperatures, individuals with antemortem fever or areas of infection (Clark et al., 1997) will also have faster rates of decomposition. Since a large number of samples used in this study were from pathological areas, it stands to reason that these areas did not present good molecular results (i.e., 6/15 pathological samples in comparison to 5/11 non pathological samples produced positive results for both mtDNA and nuclear DNA) as they would have been subject to faster rates of decomposition and increased access to microbial insults.

6.4 Endogenous DNA Preservation

The molecular approach in this particular study used a top-down format, going from essentially the ‘multi-copy’ to the ‘single-copy’ detection of DNA. By using this approach it was possible to assess the level of DNA preservation for each specimen. Mitochondrial DNA from the HVRI region (16139-16420 nps) was isolated and sequenced from all specimens except for MM1622, MM530 (except for the bone dust sample, which was identified as contamination by one of the samplers) and Whithorn. Negative results for the Whithorn sample are 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 which 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.

Early on in this project it was noticed that it was difficult to extract and analyze DNA from the Civil War samples. Problems with extracting and amplifying DNA from other Civil War bone samples were also noted by Fisher et al. (1993). In their study, mtDNA from a similar sized fragment (210bps compared to 281bps for this project) provided only 50% (4/8 undecalcified samples) and 25% (2/8 decalcified bone samples) success rates. In order to provide successful results for some of their samples, these researchers had to increase the amount of *Taq* polymerase substantially from as little as 2 U/25 µl to as much as 12.5 U/50 µl reaction. The most *Taq* Gold polymerase used in this project was 5 U/50 µl. Although Fisher et al. (1993) attribute their lack of success to inhibition, they do not provide any discussion as to what may be causing this inhibition. Their only attempt was to suggest the presence of a “bone contaminant” (*Ibid.*:67).

Since it is believed that none of the Civil War samples were ever buried, no soil contaminants (e.g., humic acid) are of issue for inhibiting PCR. Instead, information concerning their mode of conservation must be addressed. According to Paul Sledzik (personal communication) and documents found at the Armed Forces Institute of Pathology (Army Medical Museum, circa 1893), bones showing pathological lesions were cleaned, degreased and mounted dry. Cleaning involved maceration (sometimes simmering, boiling or steaming with water) of the bones and placement in copper or galvanized vessels at 100°F in order to dry the specimens. Once the tissue had been removed, the bones were degreased in benzene, often being placed in sunlight to speed the process. The document also warns that the bones must be dry prior to degreasing with benzene, otherwise benzoic acid may form which can decalcify the bones. For those

bones which needed to be ‘whitened’ due to discoloration, they were exposed to chlorine gas, chlorine water or a weak solution of chloride of lime (‘solutions thought to not corrode bone’ – stated in the document [Army Medical Museum, circa 1893]).

Of the conservation methods used at the Armed Forces Institute of Pathology, two areas are of concern for DNA preservation. The first area is the simmering, boiling or steaming in water during maceration of the specimens. Except in anaerobic environments, water easily degrades DNA via the hydrolysis of the phosphate and glycosidic bonds (Poinar, 2001). Such a procedure would greatly diminish the level of intact DNA fragments and may help explain the lack of DNA amplification or poor resolution of bases upon sequencing for several Civil War samples (e.g., MM1622 and MM530).

The second area of concern is the use of chlorine-based chemicals for whitening the specimens. Chlorine is a main constituent in bleach (Sodium Hypochlorite) which is used by *all* aDNA laboratories to degrade DNA on surfaces in the lab and/or to decontaminate tissues prior to extraction. Chloride has the same oxidizing effects on the DNA molecule as iron and thus can hinder PCR. Bleaching was most likely used on sample MM530 as illustrated by the macroscopic view of the histology section which shows a major change in coloration from endosteal (off-white) to periosteal (white) regions (see Plate A.1). This may help to explain why no DNA could be amplified from this particular specimen.

Unfortunately, several of the samples used in this project, particularly MM2880-2, MM2880-4, 1001245, 295209 and MM2510, were obtained from personal collections and

have no information concerning their method of conservation. As a result, no discussion pertaining to their conservation and correlation with preservation can occur. However, personal observation of the skull specimen from 295209 identified a shiny material covering the bone suggesting it was painted with varnish/shellac. Although this sample provided mtDNA results, nuclear DNA could not be amplified. Physical removal of consolidants has been noted to provide better quality and quantity of extracted DNA (Tuross and Fogel, 1994). Therefore, it is possible that if the material had been scraped off prior to decontamination and extraction, better DNA results could have been obtained; but the author was cautious of performing this task because of the relative thinness of the cortical area of bone for this particular specimen (remember, this was a skull fragment).

During the analysis of endogenous DNA it was noticed that not all of the tissue samples matched for two individuals. For example the humerus DNA sequence of HMC94-SK932 does not match the tooth or other long bone fragment obtained for this individual and the metatarsal of HMC94-SK1121 does not match the fibula or tibia molecular signatures. When individuals are not buried in coffins (or the coffins are not preserved), as is the case for these individuals (David Evans, personal communication), taphonomic processes tend to shift archaeological materials, especially small hand and feet bones. It is quite possible that this has occurred here, resulting in the different DNA sequences. However, one cannot rule out contamination of these two samples prior to sampling and extraction (i.e., at the excavation or laboratory where this collection is stored).

6.5 *Treponema* DNA Preservation

Since mtDNA is present in many copies in human cells, single-copy sex chromosomes were isolated via PCR to understand/correlate the different levels of DNA preservation with copy number. This was met with limited success as only 10 (with possibly 3 more) out of 32 different tissue samples could be sex-typed. With only a 31% success rate with human nuclear DNA the probability of invading microorganism DNA (which would most likely be present in smaller amounts than human endogenous DNA) detection would be quite low. However, it was unexpected that none of the samples would work.

Unsuccessful amplification of treponemal DNA in this project may be due to the inability of the organism to survive outside of its host's environment. Even within the research setting, no synthetic medium has been developed that can facilitate the growth of these organisms for an extended period of time, instead, researchers must passage it through rabbits (hamsters and guinea pigs have also been used). Experiments have recognized that *T. pallidum* survives better under anaerobic (little oxygen) rather than aerobic conditions (Wong et al., 1982). In fact, Wong and colleagues (1982:140) show that “anaerobically extracted treponemes maintained significantly higher percentage motility and virulence (shorter latent periods) than aerobically extracted treponemes.” Low survivability rates were also noted by van der Sluis et al. (1984) when they stored blood-treponeme mixtures (5×10^5 microorganisms/ml) at 4°C; after 120h of storage, infectivity was lost when rabbits were artificially injected with the mixture. Studies presented by Pusey (1933:83-4) show that within approximately 24 hours spirochetes

appear motionless (i.e., dead) and are no longer virulent when examined after the death of syphilitic patients.

Studies have also shown that *T. pallidum* is easily destroyed by heat, antiseptics, 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). The *T. pallidum* cell envelope resembles that of Gram-negative bacteria having only an inner and outer membrane (Schouls, 1992:87). Unlike most Gram-negative organisms, *T. pallidum* outer membranes do not contain lipopolysaccharides (LPS) (Schouls, 1992:91) which are known to form “a confluent and impermeable physical barrier, restricting access of lytic components” (Wilks and Sissons, 1997:171). The fragile nature of the membrane may be responsible for the lack of DNA preservation seen in ancient human remains. If the membrane cannot withstand experimental conditions, it is probable that it would not preserve in many burial conditions. Without a cellular membrane, *T. pallidum* DNA is exposed to many physical and chemical elements which can alter and destroy DNA bonds. This crucial structure may be why *Mycobacterium tuberculosis* with its thick, robust cell wall (Brennan and Nikaido, 1995; Schouls, 1992:87; 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 (Spiegelman and Donoghue, 1999).

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 the hydroxyapatite (HAP) crystals in the mineral portion of bone (Martinson, 1973; Okasaki et al., 2001). Therefore, treponemal DNA preservation is then dependent on HAP binding which must occur very soon after the death of the individual, as the bacterial membrane will be lysed by the acidic environment present during decay. This process would presumably be time and quantity dependent as there would be competition with the larger amounts of endogenous DNA being available for the binding process. Although other bacteria (e.g., mycobacteria) have developed measures to ensure DNA preservation (e.g., thick membranes), it appears (with the information presently known) that *T. pallidum* does not have any of these measures. So once the outer membrane is destroyed, the DNA has little opportunity to protect itself (except with HAP binding). This may be the case as the conservation methods and burial environments for this study have exposed the bones and constituent molecules to adverse conditions not conducive for *T. pallidum* DNA survival.

Although unsuccessful PCR results were obtained, experiments have focused on the ability to detect *T. pallidum* DNA from different environments. Research on this topic has demonstrated that DNA survivability and PCR testing has only been looked at to some degree. 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), those swabs stored dry at room temperature for 90 days (3/3), as well as punch biopsy 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 leads one to imagine that in samples retrieved from more adverse burial environments, positive amplification would be negligible to impossible.

Using more challenging variables, Villanueva and colleagues (1998) examined how certain environments (room temperature, 4°C and freeze-thaw cycles) affected the ability to isolate *T. pallidum* DNA. They were able to detect DNA (from the 47-kDa gene) in spiked CSF with 5×10^7 spirochetes/ml from all of these different environments. Although this experiment is an important one, it only looked at room temperature and 4°C storage up to 96h and only three freeze-thaw cycles. Unfortunately, these conditions (storage, freeze-thaw cycles and possibly temperature) are equivalent to few human burial environments. Experiments are needed which expose these microorganisms to longer storage times and many more freeze-thaw cycles to permit a better understanding of the DNA survival that is along the same lines as those which can be found in natural burial environments.

For those who obtain negative PCR results from clinical samples of patients diagnosed with CNS syphilis invasion, Villanueva et al. (1998:2118) say that it may be “due to the absence of organisms in the CSF, to the presence of only a minimal number of organisms, or to the presence of inhibitors to PCR amplification in the patient specimen

or patient extract.” Although this is directed to a clinical setting, it is also quite applicable to aDNA research.

It is possible that no treponemal organisms or their DNA were ever present in the samples taken for this project. When circumstances permitted, skeletal samples were chosen from both nonpathological and pathological areas to look into the hypothesis that this disease disseminates throughout the body and hence, should be found anywhere. This has been proven in clinical experiments whereby DNA detection occurred from sites of not only inoculation, but also sera and CSF (e.g., Grimprel et al., 1991; Horowitz et al., 1994; Li and Zhang, 2003; Orle et al., 1996). Evidence for the dissemination of disease pathogens has also been identified in ancient biomolecular contexts where *M. tuberculosis* DNA was detected in both nonpathological and pathological areas (Haas et al., 2000).

Regardless of the region of tissue that was sampled for this project, lack of PCR success may relate to the quantity of tissue that was sampled. On average, only 1 g of bone powder was used for extraction regardless if more was available. For Kolman et al.'s (1999) attempt (and success) at *T.p.* subsp. *pallidum* DNA isolation, 15 g of bone was used for extraction. That is *15 times* more sample than any of the cases used here, providing the possibility of *15 times* more success/probability in finding the bacterium's DNA. Therefore, the possibility exists that treponemal DNA was present in all of the individuals sampled for this study, but just not in the small quantities sampled here. Perhaps if more was sampled and extracted at the same time, PCR success may have occurred.

The possibility also exists that treponemal DNA was present, but in such a minute amount that the PCR assays could not target it. This is understandable as the treponemal assays used in this project could only detect as few as 10^3 organisms (25pg of DNA) per PCR reaction. Even in clinical experiments, detection limits are variable from as low as 20 Nichols-strain spirochetes per PCR sample (tested using 47-kDa by Kouznetsov and Prinz, 2002) to 2×10^2 organisms per reaction (377bp fragment of *polA* by Liu et al., 2001). At any rate, the levels of sensitivity achieved for this project may not have been sufficient to detect the molecules present in the samples.

It can also be hypothesized that treponemal DNA did exist in these samples, but not from the 15-kDa lipoprotein, *polA*, 47-kDa or GPD genes. Perhaps if other more conserved genes (e.g., *T. pallidum* repeat gene family [*tpr*]) had been used a positive result may have ensued due to their higher copy number. However, the *tpr* gene would not be conducive to aDNA protocols as the number of repeats are too high resulting in large amplicons (> 450 bps) (Centurion-Lara et al., 2000; LaFond et al., 2003; Stamm and Bergen, 2000). It is also possible that the individuals sampled for this project did not have syphilis and in fact suffered from another form of treponematosi (e.g., yaws or endemic syphilis) that the ARMS assays may have missed, but the other nonspecific tests should have picked up. Misdiagnosis of skeletal remains may have been even more important beyond the presence/absence of treponemal diseases. With further investigation, historical documents for MM2880-2 and 4 (Army Medical Museum, 1868), 1001245 (Otis, 1868a) and MM530 (Lamb, nd) suggest that these individuals may not have been suffering from syphilis at the time of their death.

The final suggestion of Villanueva and colleagues (1998) that inhibitors may be present in the specimen extract is quite possible in this situation. Mitochondrial DNA isolation from HVRI was successful in most of the specimens except for MM1622, MM530 and Whithorn. When it was realized that many of these samples did not work on the first try with the standardized method, inhibition was suspected. Inhibition was overcome in some instances (e.g., with samples 295209np, HMC94-SK805 fibula path) when more BSA, MgCl₂ and/or *Taq* Gold were added to the PCR mix. In another attempt to check this phenomenon, each of the unsuccessful samples were spiked with 1µl/2.5µl of positive control DNA (K562 Invitrogen) and only MM1622 femur and Whithorn appeared to be inhibited as no/very light band was present (see Figure 5.2). However, many of the bands in the gel were lighter than expected, which suggests that some inhibition may be playing a part in the negative results obtained. Lack of inhibitors in the extract of individual MM530 is another testament to the probable lack of any preserved DNA within this sample due to bleaching. The absence of primer-dimers in some of the amplifications suggests inhibition of the PCR as well. This was most apparent when the inhibition test was performed for those samples that failed to provide DNA results (see Figure 5.2).

The last possibility to discuss regarding the lack of *T. pallidum* DNA within these individuals is the theory that by the time bone lesions manifest, no bacteria (and hence, no DNA) exist at the site of infection. Through different types of research it has been noted that by the tertiary stage (where the majority of bone lesions develop), 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; Musher and Baughn, 1998; Schell and Musher, 1983; Smith, 1976) possibly due to the degraded remnants of the bacteria at that particular site (Jaffe, 1972:928; Resnick and Niwayama, 1995:2427) or to treponemal antigens (Salazar et al., 2002). This was first suggested by Schneider (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 as Cotran et al. (1999:84) describe granulomatous inflammation (hallmark of syphilis infection), as

a specific type of chronic inflammatory reaction characterized by accumulations of modified macrophages (epithelioid cells) and initiated by a variety of infectious and noninfectious agents. The presence of poorly digestible irritants, T cell-mediated immunity to the irritant, or both appears to be necessary for granuloma formation.

A key section in this passage is that it can be “initiated by a variety of infectious...agents” meaning that the organism had to be there at some point to start the reaction off. This suggests that treponemal DNA has the ability to exist at this site if it is not used or cleared by the host. However, studies have been able to show that dead *T. pallidum* are rapidly cleared from the blood (Wicher et al., 1998), implying little possibility for DNA survival. It must also be remembered that bone lesions can be evident as early as the secondary stage. This stage is known to be highly infectious with individuals teeming with

spirochetes. As a result, the possibility still exists that treponemal DNA can be isolated from skeletal remains displaying osseous responses to disease, although distinguishing between individuals with secondary or tertiary lesions may be problematic.

6.6 Rabbit Infection

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 the treponematoses are tested using harvested spirochetes 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; Wicher et al., 1998). Results obtained in this project are in accordance with others (Wicher et al., 1998:2509) in that DNA could still be isolated from the site of injection in the rabbit with persistent infection (refer to Figure 5.7) suggesting that the “process of elimination of *T. pallidum* from primary sites of infection is prolonged and incomplete”. In a non-molecular approach (i.e., organisms were looked for) Frazier et al. (1952) were still able to demonstrate the presence of spirochetes in testes tissue and blood of rabbits 31-42 months post inoculation via dark-field preparations. 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 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 remains slim.

Isolation of treponemal DNA from rabbit bone was successful (refer to Table 5.5 and Figures 5.7 and 5.8), however it could only be found in the acute stage. It is believed that this is the first time bone has been used as a clinical source of DNA for syphilitic amplification reactions. Such an 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 in this project. 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 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 amplicons could be isolated when targeting treponemal DNA within the bone samples used in this project.

CHAPTER SEVEN:

CONCLUSIONS

Since the development of the three main hypotheses to explain the origins of syphilis, evidence for and against them has been presented. Skeletal evidence from recent excavations in Europe has pushed back the clock concerning the pre-Columbian presence of syphilis in the Old World. As excavations continue around the world, it is believed that more evidence will be garnered that will clearly show the presence of syphilis or treponemal precursors prior to 1492. With technological advances, such as PCR and DNA sequencing, whole genomes of the *Treponema* genus will also be available for phylogenetic comparison. This will provide the data and methods necessary ultimately to test what relationship exists among the four human pathogenic treponemes.

When this project was designed, I believed that using samples from the Civil War time period from the Armed Forces Institute of Pathology, Washington, D.C., was a critical feature because medical diagnostic notes showed the presence of syphilis. With positive identification of the presence of this disease, it was assumed that these individuals would provide an established diagnosis and serve as a positive control. However through this analysis, it appears that some of the individuals suffered from another disease (e.g., tuberculosis) and not syphilis. As some of the samples were donated, their life history could no longer be considered accurate as putative diagnoses could only be made via the select bones which were kept, thereby mimicking the shortcomings of the paleopathological approach. These samples also turned out to be problematic because the measures used to conserve the bones (boiling, exposure to

chloride) were not conducive for DNA survival. Even though the Civil War period samples were the youngest by date in this project, they were the worst preserved, both by histological and molecular standards. This experience has taught me to look and ask for more detailed information concerning samples, their history and their mode of conservation prior to exposing them to DNA analysis. Although this appears to be a logical approach, it is easy to be blinded by the other qualities that a sample holds, such as the presumed accuracy of a medical diagnosis.

The samples from the United Kingdom and Belleville, Ontario were a different story. With the exception of HMC94-SK805 and HMC94-SK1121, these individuals provided classic macroscopic alterations of caries sicca, a combination of proliferative and destructive processes and multiple bone involvement indicative of venereal syphilis. Interestingly, these two exceptions (along with HMC94-SK1216 and Belleville B302) were the samples that provided clear microscopic indications of syphilis via the presence of Polsters and Grenzstreifen. These samples (except for Whithorn gumma) also provided either mitochondrial and/or nuclear DNA results suggesting well preserved bone constituents that were confirmed with histological analyses.

Applying several PCR techniques (i.e., mtDNA, amelogenin and bacterial) to these skeletal samples (with limited success) shows the different levels of DNA preservation. Also, although negative results were obtained from my attempt to isolate *Treponema* DNA, multiple assays were used instead of the more common single assay approach, with each one showing negative results. Failure to obtain treponemal DNA does not necessarily mean the organism is absent, but instead may point to PCR protocols

which are not sensitive enough to prime off the minute quantities of bacterial DNA that may be present in bone. Misdiagnosis of the skeletal pathology is another possibility.

PCR use and its success in a project of this sort are based primarily on the presence/absence of treponemal DNA in bone. When positive results were not forthcoming, it was decided to investigate experimentally the presence of treponemes in bone at different stages of infection by using the rabbit model. This important experiment revealed that the bacterium does indeed reach the bone, but only in the acute stages of pathogenesis. The inability to isolate syphilitic DNA in rabbit bone beyond the early stages of the disease by means of the protocols set forth in this project suggests that there may be a temporal difference in the extent of organism invasion during various stages of syphilis. This experiment helps explain the failure to detect positive PCR results from the human remains studied in this project as treponemal DNA could only be isolated in acute forms of rabbit syphilitic infection and not from later stages of the disease which is more indicative of osseous involvement in humans (and hence when paleopathological identification is possible). It may also provide new lines of evidence regarding skeletal involvement in syphilis. That is, it is not the actual bacterium that is causing the skeletal alterations, but instead remnants of *T. pallidum* and/or an allergic or immunopathologic response to those remnants by the host.

Bone alterations allow paleopathologists to identify disease in the past. However, some manifestations mimic several diseases, like syphilis and tuberculosis. When uncertainty arises in the diagnosis it is recommended that a microscopic approach be used to decipher the disease process. In fact, microscopic analyses served to complement the

macroscopic identification of syphilis in the historic skeletal samples from England and Canada used in this study. Diagnosis was based on parameters presented by Schultz (1994, 2001 and 2003), which characterize syphilis at the histological level. Observation of the micro-architecture for several of the samples used in this project allowed for a more comprehensive approach regarding the identification of disease (processes) in ancient human remains. In most samples Polsters and Grenzstreifen, or remnants of such structures, could be identified suggesting the presence of a chronic, inflammatory disease such as syphilis. Sinous lacunae were also observed in all histological samples pointing to lytic actions (osteitis). The combination of both proliferative and destructive processes is pathognomonic for syphilis and lends to a more accurate diagnosis of this infectious disease within these individuals.

It must be pointed out that a diagnosis of syphilis was supported at the microscopic level for several individuals (e.g., UK HMC94-SK805, HMC94-SK1121, HMC94-SK1216 and Belleville B302), assuming that the variations observed are in line with what may be expected. This statement should be considered provisional because the paleohistopathological literature (which is primarily based on active forms of the disease) does not provide a good guideline for variation in Polster and Grenzstreifen structural formations, especially variation at different stages of infection (i.e., acute or chronic) or even development (e.g., active, latent or healed). Observations in this project revealed these histological structures to be focalized, variable in size and shape and sometimes altered or hidden by the dynamic nature of bone remodeling. More studies that use bone

microscopy to identify disease, particularly treponemal diseases, will eventually fill this void and provide a better database for comparison.

The microscopic approach also provided information concerning the preservation of histological structures. Diagenetic alterations created a lack of birefringence (and hence poor preservation) in the samples that was likely caused by microorganism and possibly fungal invasions as well as alterations (e.g., hydrolysis, oxidation) in the collagen/hydroxyapatite matrix. This study agreed with others that note a correlation between well-preserved bone micro-architecture and (good) molecular results. Poor DNA results may be related to the less than optimal conservation methods applied to some of the samples and the presence of pathological lesions.

It must be noted that no single factor can determine the level of preservation of bone and its histological and molecular constituents. Complicated interactions between many different variables within the burial environment, both extrinsic and intrinsic to the individual, and even prior to inhumation, are at play. Among these variables, one must be concerned with environment stability, soil type, size of bone and presence of disease. Although there are techniques that help to decipher DNA preservation (e.g., HPLC, amino acid racemization, C/N ratios, etc) prior to extraction and analysis, they can be costly, time consuming, require specialized equipment and can degrade precious material. To compensate for these factors, if a bone histology lab already exists in the anthropology department (the other techniques and equipment are not normally), microscopic screening would allow for a relatively quick and inexpensive process for providing an optimized idea of DNA preservation and its potential recovery, as it did in this project. It is an

invasive technique, but the bone is not destroyed and can be reburied or used for other histological analyses at a later date.

It was hoped that by using the existing PCR protocol (Kolman et al., 1999) and providing a novel PCR approach, i.e., the Arms Refractory Mutation System (allele specific PCR) to isolate and identify syphilis in historic skeletonized individuals, that this project would add new confirmed cases of the infectious disease in the past to the aDNA repertoire. This project was not successful in extracting and amplifying treponemal DNA either from samples with documented proof or even from putative displays of syphilis suffering, but it was successful in all of its other objectives.

1. This project benefited from a multi-component analysis to help in the identification of syphilis. Although aDNA methodologies were negative, macroscopic and microscopic observations of the material pointed to confident putative identification of syphilis in nine individuals: Civil War 1002700, MM1154, UK Whithorn gumma, Blackfriars SK77, HMC94-SK805 (co-identified using microscopy), HMC94-SK932, HMC94-SK1121 (co-identified using microscopy), HMC94-SK1216 (co-identified using microscopy) and Belleville B302 (co-identified using microscopy).
2. Confident microscopic evidence of syphilis in several individuals (UK individuals HMC94-SK805, HMC94-SK1121, and Belleville B302) in this project ultimately increases the paleopathological repertoire which can be used as a basis of comparison for future histological studies. It also illustrates the fact that a range in variation for both size and shape does exist in the presentation of the

characteristic structures (e.g., Polsters and Grenzstreifen) associated with treponemal infection.

3. Although some exceptions did occur (e.g., UK Blackfriars SK77), a correlation between good osseous micro-architecture preservation and positive DNA results seemed to be the norm. For example, the samples from the Civil War MM530 individual had no birefringence or osteon structures preserved and also provided no molecular results.

4. Human mtDNA and amelogenin DNA were both isolated from samples from Civil War individuals 1002700, MM1154, and MM2510, UK individuals HMC94-SK805, HMC94-SK932, HMC94-SK1121, and Blackfriars SK77 and Belleville individual B302, ultimately showing that a number (8/13) of the individuals used in this project had well-preserved endogenous multi-copy and single-copy DNA.

6. The inability to isolate treponemal DNA from any of the human samples used in this project is in part related to the fragile nature of the *Treponema* organism which appears to reduce its longevity in bone both within the living host and the burial environment. The rabbit model experiment performed in this project also provides another explanation as to why treponemal DNA could not be amplified and is an important contribution to the debate of whether the bacterium reaches the bone and remains there in later stages to cause bony lesions.

Although no molecular evidence was detected to support or deny any of the theories pertaining to the origins of syphilis, especially the Unitarian and the pre-

Columbian, histological evidence does suggest it was present in England prior to 1492. This secondary form of evidence helps support the macroscopic identification of syphilis and shows the power of a multi-methodological approach to the identification of disease in the past. This thesis also serves as a warning to those who think aDNA can provide all of the answers to unraveling patterns of disease in the past. Only with advancements in science will we gain a better understanding of the microbiology of organisms that cause disease and perhaps it will help to resolve the origins and evolution of not only syphilis, but also other diseases.

7.1 Future Work

The lack of success in isolating treponemal DNA from archaeological human remains in this project suggests many avenues for future research. First, it is important to use other methodologies to ascertain the preservation of not only histological structures, but also DNA. For example, observing histological sections under a fluorescent microscope will reveal areas of demineralization and/or areas of lost birefringence (Grupe et al., 1993). Such an approach will provide more information on the state of mineralization, and inadvertently DNA preservation, of the bones used in this project. Other measures, such as HPLC (high pressure liquid chromatography), mass spectrometry, amino acid racemization and/or C/N ratios should be employed to check protein preservation of the samples. Since the bond strength between proteins is similar to DNA binding, it can be used as a relative measure of DNA preservation and has been suggested by some as a prerequisite for aDNA analysis (Cooper and Poinar, 2000). When these methodologies find little preserved protein, other samples should be chosen for

analysis so that time and money is not wasted. Also if little protein is discovered, but DNA results are positive, contamination is an issue.

Although it was believed that the pathological lesions identified on those samples taken from the Civil War collections at the Armed Forces Institute of Pathology were caused by syphilis, poor documentation of this fact may have been at issue for some of the specimens (i.e., for those specimens which had been bought or donated by private collectors). To increase one's odds of using truly syphilitic samples in aDNA analyses, it may be advisable to use specimens from those reference collections with better documentation (e.g., anatomical collections), even though these collections also undergo rigorous cleaning which may limit the preservation of DNA. However, this would increase the possibility that the samples are actually positive for syphilitic infection.

It was discovered after the fact that several of the individuals analyzed here may have suffered from another disease than syphilis or had a co-infection. Syphilitic bone lesions can mimic those of many other infectious diseases contributing to misdiagnoses. Of importance for any project would be to first attempt to isolate mycobacterial DNA in the forms of tuberculosis (e.g., using Taylor et al., 1996) and leprosy (e.g., using Rafi et al., 1994) as these diseases cause similar alterations in bone to syphilis. Positive results from these tests, especially tuberculosis, would indicate that the individual either suffered from this disease or had been in contact with it at some point in their life.

Finally, altering the assays used in this project so that more powerful PCR systems can be developed is an important area of future research. This includes increasing sensitivity levels so that fewer numbers of organisms are needed for a positive result.

This may entail using additives in the PCR reaction (e.g., DMSO, BSA) or during extraction and purification (e.g., PTB) to help overcome inhibition. Increasing cycle numbers or trying different extraction protocols may also assist in isolating bacteria and eliminating inhibitors or contaminants. It would also be wise to redesign the primers so that smaller amplicons can be produced. Shorter sequences are known to preserve better. Finally, using fluorescently labeled primers will also increase the sensitivity of the amplification system. All of these approaches may optimize the possibilities of isolating treponemal DNA for fellow researchers. If the low numbers and fragile nature of the spirochete can be overcome by future advances, either by ameliorating extraction and amplification procedures as well as by choosing better samples, we may be able to unravel the origins of the treponematoses.

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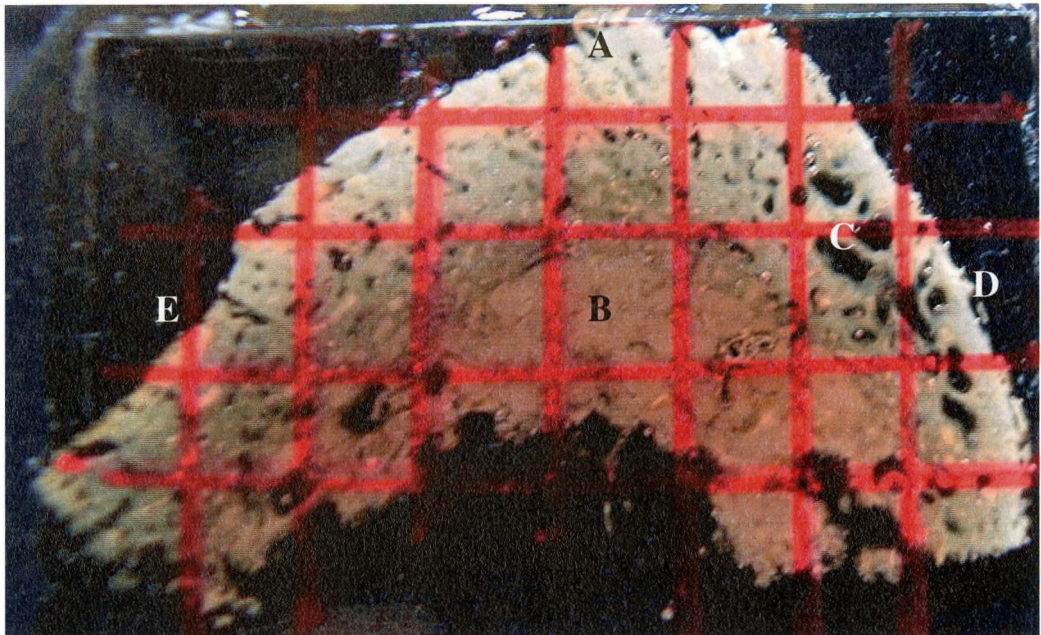
APPENDIX:**MACROSCOPIC PICTURES of HISTOLOGICAL SAMPLES**

Plate A.1: Macroscopic view of histological section for Civil War MM530 femur. Note the change in shading/coloration of bone from periosteal surface to inner compact bone. The bone is abnormally thick for a femur.

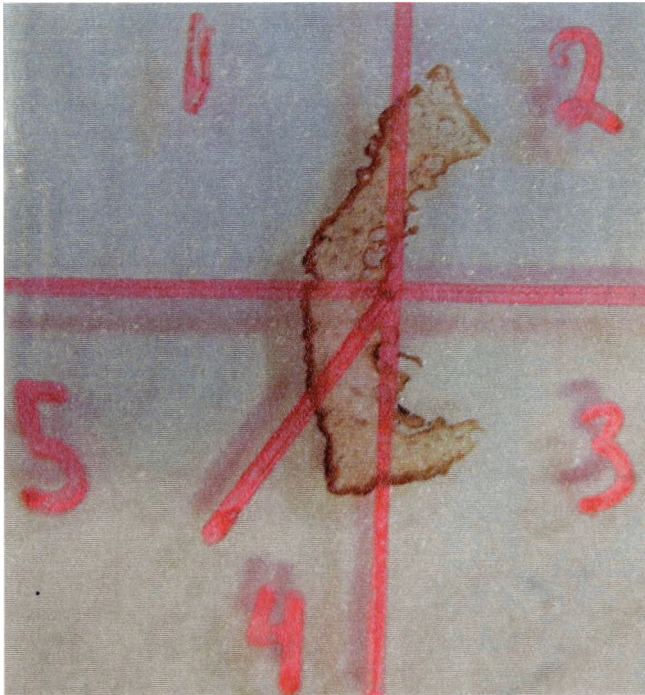


Plate A.2: Macroscopic view of histological section for UK HMC94-SK805 fibula. Note the red/brown staining of external surface of bone.



Plate A.3: Macroscopic view of histological section for UK HMC94-SK932 proximal right humerus.

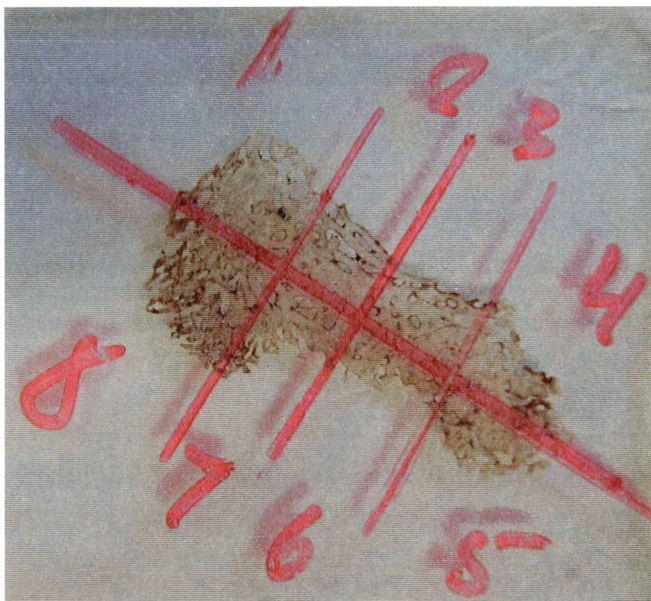


Plate A.4: Macroscopic view of histological section for UK HMC94-SK932 long bone pathological fragment.

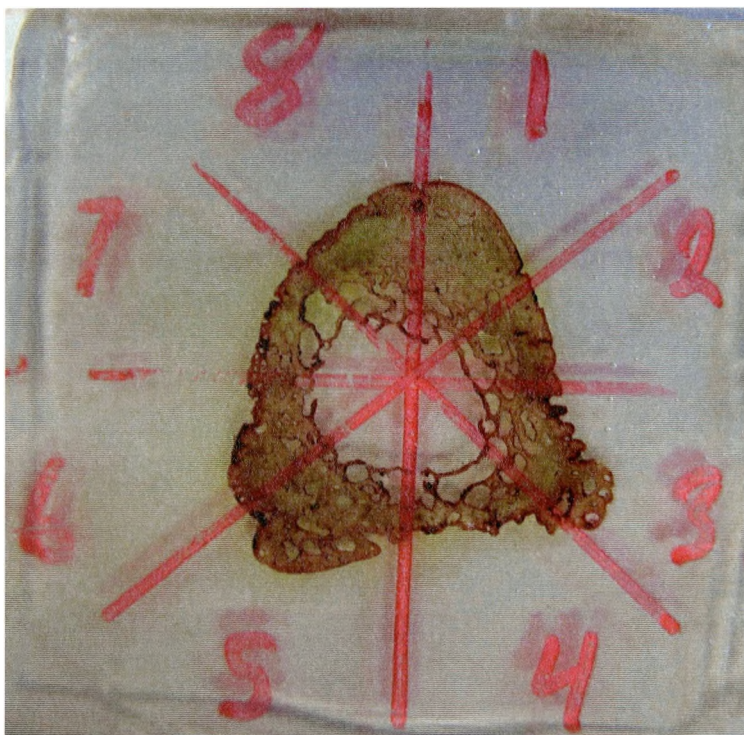
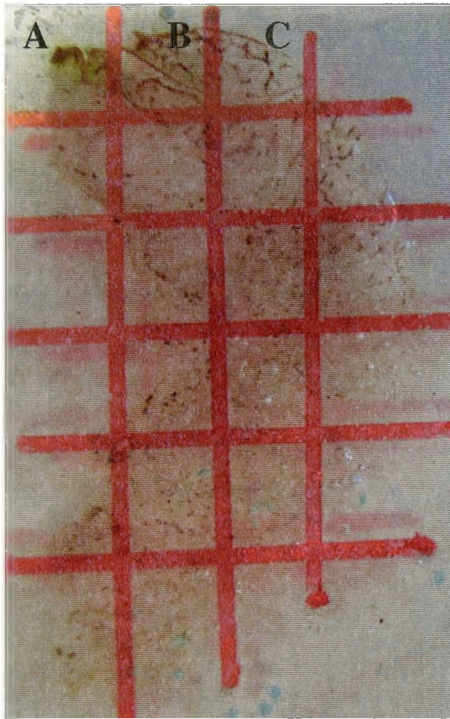


Plate A.5: Macroscopic view of histological section for UK HMC94-SK1121 fibula. Note the presence of staining on the periosteal surface and presence of abnormal bone growth most notable in sections 3 and 5

**Plate A.6:**

Macroscopic view of histological section for UK HMC94-SK1121 tibia (sample taken from upper mid third of shaft – area of osteitis). This sample is taken from the upper mid third of the shaft where an area of osteitis had been observed. See plate of bone where sample was taken from.

**Plate A.7:**

Macroscopic view of histological section for UK HMC94-SK1216 tibia fragment 1

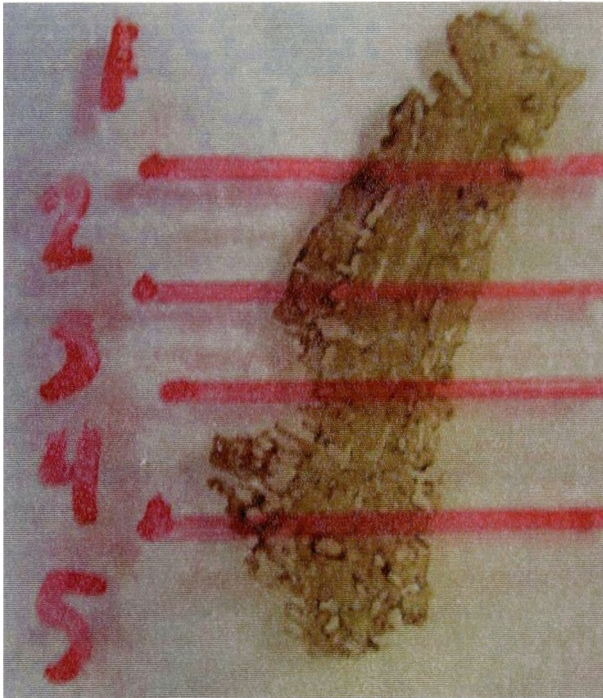


Plate A.8:
Macroscopic view of histological section for UK HMC94-SK1216 tibia fragment 2.



Plate A.9: Macroscopic view of histological section for UK Blackfriars SK77 rib. Macroscopic view of the histological section reveals that the normal osseous formation of dense cortical bone enveloping lots of trabecular bone normally present in ribs is altered in this individual/sample due to a pathological process. The upper right of the slide shows the replacement of dense cortical bone with aeriated trabecular bone (with spicules). A destructive/lytic process has destroyed the cortical bone and has replaced it with spicules of trabecular bone.

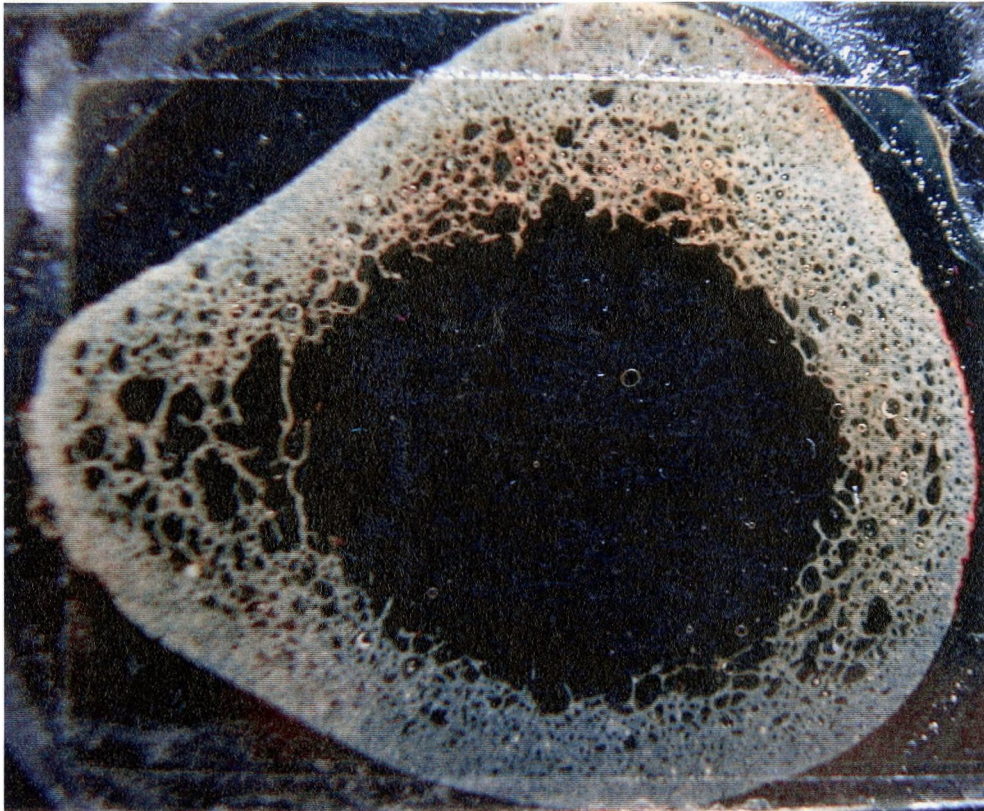
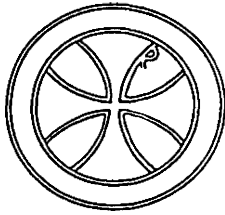


Plate A.10: Macroscopic view of histological section for Belleville B302 femur (section taken just inferior to pathological area, hence the reason why there are no obvious areas of pathology).



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30/04/04

Dear Tanya von Hunnius

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I wish you every success with your publication.

Yours sincerely

Janet Butterworth
Business Manager