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PALEOEPIDEMIOLOGY OF BACTERIAL INFECTIONS AMONG PREHISTORIC HUMAN POPULATIONS IN NORTHERN CHILE:
AN ANCIENT DNA APPROACH

by

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ABSTRACT

Paleoepidemiology of Bacterial Infections among Prehistoric Human Populations in Northern Chile: An Ancient DNA Approach

by

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Bacterial pathogens not primarily affecting the skeleton but causing sepsis and death, have not been systematically studied in prehistoric human populations, although increasing evidence support our species long co-evolution with many of them. With molecular methods we can identify bacteria at the species level and distinguish pathogenic from environmental and soil bacteria. Bone marrow of ancient people may provide valuable information about ancient pathogens causing sepsis and death. To test the hypothesis that PCR amplification using universal bacterial primers will identify prehistoric bacterial pathogens in bone marrow, 30 samples were aseptically obtained from partially mummified remains of three archaeological sites of Northern Chile (Mo-1-6, AZ-140, and LLU-54) dated between 600 - 4,500 years B.P. Eight of the 30 samples (27%) yielded human DNA sequences documenting DNA preservation, three of which also amplified bacterial 16s rDNA, none corresponding to human pathogens. It appears that contaminant DNA prevents the detection of ancient pathogens with this method.
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Professor Daniel Benyshek did not hesitate in accepting me as his student when Professor Arriaza was appointed full professor at the Universidad de Tarapacá in Chile. Professor Benyshek patiently walked me through the process of writing a thesis,
emphasized the importance of maintaining a theoretical thread in scholarly inquiry, and was an invaluable discussion partner.
CHAPTER 1

INTRODUCTION

"The past has much to teach us about the factors contributing to the emergence of disease, if we can successfully address the challenge" (Ubelaker 2003).

Traditionally, infectious diseases have been approached using military analogies, where pathogens are the enemy to be sought out and destroyed. The success of biomedicine’s use of antibiotics during the 20th century reinforced the notion that infectious diseases could be defeated. The past two decades, however, have witnessed an increase in “emerging” infectious diseases-- a surge of antibiotic resistant bacteria with a consequent increasing number of ineffectively treated infections-- and the re-emergence of bacterial diseases we once thought defeated in industrialized nations. Voices are emerging from the fields of microbiology, immunology, and anthropology for the need of radical new strategies to study and fight infectious disease. In a recent Nature editorial entitled “the biology of infection” referring to an original article describing the first new chemical class of antibiotic to be found in more than two decades (Nature 2006), the author advocates for a more holistic approach to fight infectious disease incorporating the complex set of feedback loops between pathogens, host immune system and our own microbiota. A truly holistic approach, however, needs to incorporate an evolutionary perspective into these loops. How can we pretend to understand the biology of present human bacterial infectious disease without incorporating the evolutionary trajectories of
the human host and its pathogens? The genus *Homo* of the Primate Order, represented today by a single species, *Homo sapiens*, is the most widely distributed on the planet. The earliest *Homo* appears first in the fossil record in Africa and soon began a journey out of Africa around two million years ago. By 14,000 years ago *Homo sapiens* had reached every major landmass except for Antarctica. In a Darwinian view, environmental change has played a mayor role in shaping life on Earth by means of natural selection. The geographically patterned phenotypic diversity of the human species is in part the result of Darwinian evolution. Remarkable for *Homo* though, has been the ability to adapt to the different environments encountered in the migration out of Africa beyond natural selection, by means of culture. Human bio-cultural adaptations across time and space must have acted as selective forces in the plethora of microorganisms that compose our bacterial microflora, resulting in new adaptations. Some of these bacterial adaptations may well have contributed to an increased virulence for the human host. Unraveling the host-pathogen co-evolution will provide answers on the when, how and why bacterial pathogens have emerged in the past.

Of the multiple bacterial pathogens that afflict the human species, those reproducing primarily in humans have persisted or are re-emerging as significant public health problems (e.g. *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Haemophylus influenza*, and *Treponema pallidum*). In contrast, bacterial pathogens that afflict humans as a result of exposure with an animal reservoir through a vector or the ingestion of a contaminated source, have shown to be easier to control with public health recommendations, e.g. rodent control for *Yersinia pestis* (bubonic plague), milk
pasteurization for *Mycobacterium bovis* (tuberculosis), and egg refrigeration for *Salmonella enteritidis* (diarrhea) to provide a few examples.

Bacterial pathogens, with humans as their primary host, are all distributed worldwide. Although some may choose to ascribe their world distribution to the post-Columbus era, as a result of a recent global human network, multiple lines of evidence support a long co-evolution with their human host. Applying a co-evolutionary perspective to the study of human bacterial pathogens could shed light not only on the emergence of virulence in relation to human adaptations but also on common trajectories across time and space. Even more, we may be able to determine if any of our bacterial pathogens are legacies from the cold adapted Neanderthals. The place of Neanderthals in modern human origins is still being debated in anthropology. Genetic evidence supports an extended separated evolution until the reunification with later migrating modern humans into Europe. Mitochondrial DNA sequences of Neanderthals are not represented in modern populations, and this is cited as critical evidence for a lack of interbreeding, although some interbreeding cannot be excluded (Serre et al. 2004, Caramelli et al. 2003, Kings et al. 1997). Despite little if any evidence for interbreeding between Neanderthals and modern humans, exchange of microflora between the two during several thousand years of shared geography is highly possible.

The co-evolutionary perspective is needed in the fight against infectious disease because in spite of intense research efforts developing vaccines and an impressive number of antibiotics, bacterial infectious diseases remain a major global human health challenge. These infectious organisms, not surprisingly, simply continue to adapt to conditions that threaten their survival, as they have done since the beginning of life on
Earth for over three billion years. The fields of paleopathology and paleoepidemiology can reveal the co-evolutionary struggle between ancient human populations and their pathogens, contributing to our understanding of the role played by human bio-cultural adaptations in the evolution of virulence and the emergence of epidemics in the past. The most direct evidence for the human disease experience in the past originates from the remains of the people themselves (Ubelaker 2003), and significant advances have been made for diseases such as tuberculosis and treponemal disease that leave traces in the skeleton. In contrast, very little is known about bacterial diseases in prehistoric populations that do not primarily affect the skeleton. The aim of this thesis is to explore the utility of molecular biological methods in determining which of the bacterial pathogens that leave few---if any---traces on the skeleton afflicted past populations.

Theoretical Orientation

Epidemiologic transition theory first proposed by Omran (1971) and further expanded by Barret and collaborators (1998) proposes a Paleolithic baseline for infectious diseases with small nomadic bands of foragers, followed by three epidemiologic transitions, each of them marked by changes in human behavior. The first epidemiologic transition occurred during the Neolithic revolution. Bio-archaeologists have documented an increase of infectious disease rate everywhere at the time of the adoption of agriculture or pastoralism by prehistoric populations (Cohen and Armelagos 1984). The second epidemiologic transition corresponds to industrialization, roughly mid-nineteenth century Europe and North America, with a shift from infectious to chronic disease mortality. By the end of the twentieth century, the process of
globalization led to the third epidemiologic transition, with a resurgence of infectious
disease mortality (Barret et al. 1998). Fenner (1980) further proposes a linear model in
which there is an increase of population density and an increase of infectious disease
load over time. Using this linear model, and given the exponential population growth
continuing in the modern era, the Fenner model predicts a continuous increase of
infectious disease load for future generations.

Darwinian Medicine is a relatively new interdisciplinary field that brings together
physicians, biologists, anthropologists, psychologists and others, whose aim is to seek
evolutionary explanations for the vulnerability of modern humans to disease. In other
words, it studies medical problems in the light of evolutionary processes.
As observed by Nesse and Williams (1991), evolutionary medicine teaches us that
disease does not result from malevolent and random forces, but from a historical process
of natural selection.

In retrospect, it is now clear that health scientists were overly optimistic about the
eradication of infectious diseases by the mid 20th century. The introduction of antibiotics
and the implementation of vaccine strategies did significantly reduce morbidity and
mortality due to targeted pathogens. Notably, however, the only truly eradicated
infectious disease by the turn of the twenty-first century was smallpox. Antibiotics have
not eliminated any bacterial disease, and their inappropriate (over)-use has caused the
emergence of multi-drug resistant bacterial strains, particularly staphylococci,
streptococci and enterococci, which are increasing health threats worldwide. Today,
many health scientists are seeking to integrate germ theory with evolutionary principles,
emphasizing the influence of environment and human behaviors and activities in
determining the characteristics of infectious agents. As a consequence, evolutionary medicine has recently witnessed a shift of paradigm in relation to the evolution of virulence. The prevalent dogma before this shift maintained that disease organisms should evolve towards a benign coexistence with their hosts. Consequently, harmful infectious diseases represented a transitory state of maladaptation, as proposed by Dubos (1965) and Burnet and White (1972), and therefore most pathogens were perceived as having entered human populations in the recent past. To exemplify this point, the virulence of tuberculosis among American Indians was attributed to a recent association between Mycobacteria and humans in the New World. Anthropologists, through paleopathological research, have played a pivotal role in challenging this notion, however, demonstrating that tuberculosis has had a worldwide distribution for thousands of years. These discoveries showed that the traditional ‘benign coexistence’ viewpoint failed to incorporate evolutionary mechanisms such as natural selection over the short run, and instead focused on what was stable over the long run (Ewald 1994).

A major advocate for the shift to the new evolutionary paradigm has been Paul Ewald (1991, 1994, 1998 and 2003). Although the modern evolutionary perspective does not dismiss the notion that virulent pathogens may be harmful-- in part due to their recent acquisition among humans-- it emphasizes that virulent vector-borne pathogens may be maintained indefinitely in human populations. As an example, we could site malaria plasmodiae that have been virulent and have caused deaths to humans since antiquity. Anthropologists have proposed that inverse transhumance (flock movement to higher elevations during the summer) in the island of Sardinien (Brown 1981) and the increased incidence of multiple inheritable hematologic diseases, including sickle cell
anemia, are human adaptations at the cultural and biological level, respectively, resulting in improved Darwinian fitness of past populations. Nevertheless, they have not resulted in a reduction of the plasmodia virulence.

Other implications of a long-term use for evolutionary biology have been the possibility of manipulating the evolution of infectious diseases towards harmless ends. With the possibility of virulence management through public health measurements, the virulence of infectious diseases gained respect as a topic for evolutionary study. The dominant concept behind most arguments of virulence management is a rapid response to indirect selection based on a trade-off model developed by Anderson and May (1982) and Ewald (1983). This adaptive model suggests that the level of virulence of a parasite is a consequence of its evolutionary adaptation to maximize its total transmission from infected hosts (Anderson and May 1982). As noted by Ebert and Bull (2003) however, there are several difficulties with empirical applications of the trade-off model. For example the faulty intuitive idea that reducing the opportunities for parasite transmission would favor lower virulence because high virulence would kill the host too quickly. The foundation of virulence management is that virulence will evolve in response to indirect selection, meaning that changes in opportunities for parasite transmission will select for changes in virulence, but the model does not address the impact of covariance between virulence and transmission. The application of multiple models has shown that no single factor is likely to be responsible for the differences in virulence of different parasites (Ganusov and Antia 2003) and that direct selection against virulence itself might be a more rewarding approach to managing the evolution of virulence (Ebert and Bull 2003).
With the advent of molecular techniques and solid advances in ancient DNA research we are given a window of opportunities to study disease emergence and evolution in past populations. Scholars have been able to document pathogens’ DNA in skeletal lesions of tuberculosis (Salo et al. 1994, Arriaza et al. 1995, Braun et al. 1998, Konomi et al. 2002) and treponematosis, although for the latter only one case has succeeded due to the very rare presence of treponema in skeletal lesions (Kolman et al. 1999). Phylogenetic and ancient DNA analysis of Mycobacteria has shown that instead of humans acquiring tuberculosis from cattle during their domestication in Neolithic times, changes in human behavior (settlement patterns, crowding) were most likely responsible for an increase of disease prevalence becoming visible in the archaeological record. Therefore, instead of humans acquiring tuberculosis from cattle, it seems that cattle may have acquired it from us (Brosch 2002). These recent discoveries are important because they demonstrate that many of our assumptions about the origins of disease as well as the evolution of virulence are wrong and that some human pathogens have a tremendous antiquity as part of the human experience.

Today, the advent of ancient DNA methods opens up the opportunity to research infectious diseases that do not primarily afflict the skeleton, but can be found in soft tissues (rarely preserved), and in the bone marrow or dental pulp cavity if bacteremia occurred before death. The focus of the present thesis will be on bacterial diseases that do not primarily afflict the skeleton, and have therefore not been directly addressed in paleopathology or paleoepidemiological research.
Purpose of the Study

A first step towards understanding host-pathogen co-evolution among humans is to determine what bacterial pathogens afflicted people in prehistoric times, and secondly, to establish how far back in time the association begins. This information then might provide a foundation for future research that may be able to determine the changes that a given pathogen has undergone in relation to human bio-cultural adaptations and migrations. Understanding these human-host/pathogen relationships and time scales is important because it will help calibrate bacterial evolutionary rates. Presently, bacterial mutation rates have been estimated calibrating with the possible emergence of the mammalian gut some 100 million years ago, a rather arbitrary timescale (Ochman et al. 1999). If we are able to provide a temporal frame to pathogens evolutionary changes by means of aDNA research we will be able to test models of virulence evolution and emergence of epidemics. Our present cultural practices may be drastically influenced by the knowledge emerging from research in this area. What form of human interactions will keep the global infectious disease burden low with a doubling of the current human population that might be expected by the year 2050?

Specific Research Hypothesis

Paleopathological research has shown at the macroscopic level that a large proportion of the populations in northern Chile suffered from pneumonia, based on findings on mummified individuals (Aufderheide et al. 2002, Allison 1984). Clearly pneumonia was a common disease in humans of the South Central Andes for the past several thousand years. This applies to populations living at the Pacific coast and western valleys. Was the
etiology of pneumonia in prehistoric times the same as the one recorded in historic times before the introduction of antibiotics and vaccines? Or is the high pneumonia prevalence the product of a unique local constellation? Multiple lines of evidence support a long coexistence between humans and commensals of the upper respiratory tract such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, the two major contemporary etiologic agents of pneumonia worldwide. Commensals are one of two partners living in permanent close association, which gains a slight benefit from the association without causing serious disadvantage to the other. Certain strains of these two bacterial species, however, are characterized for being able to cause invasive disease such as otitis media, meningitis or pneumonia, leading to sepsis and death if untreated. *Streptococcus pneumoniae* and *Haemophilus influenzae* both exhibit a very large number of serotypes and a geographically patterned distribution that attest to a long coexistence with the human species, as variation accumulates over time. Additionally, *Streptococcus pneumoniae* and *Haemophilus influenzae* are the two major bacterial pathogens that colonize the nasopharynx of contemporary humans, and continue to be a significant cause of morbidity and mortality despite vaccination strategies and antibiotic treatments. Both pathogens exhibit increasing antibiotic resistance worldwide, and a shift to non-vaccine invasive serotypes, in areas where vaccine strategies have been implemented, is emerging (Kyaw et al. 2006). A better understanding of the co-evolutionary history of ancient pathogens and their human hosts over time is likely to prove invaluable in understanding the processes of infectious disease emergence and evolution.

In order to explore whether or not commensals of the upper respiratory tract were a significant cause of morbidity and mortality in prehistoric sedentary populations, such as
those of Northern Chile, from 4,500 to 600 years B.P., this research will test the following hypothesis:

1. Research Hypothesis: The use of universal bacterial primers for PCR analysis of ancient DNA, extracted from bone marrow samples will positively identify human pathogens of prehistoric populations.

2. Null Hypothesis: The use of universal bacterial primers for PCR analysis of ancient DNA, extracted from bone marrow samples will not prove to be a useful method to identify human pathogens of prehistoric populations.
Anthropological research has used a variety of approaches in the study of infectious disease. In an extensive review of the anthropology of infectious disease Marcia Inhorn and Peter Brown (1990) found biological, ecological, and sociocultural approaches as the three major avenues explored by anthropologists in this pursuit. Infectious diseases have provided a rich area for anthropological research as human populations have been forced to adapt to infectious agents on the levels of both genes and culture. Many scholars argue that infectious agents have profoundly affected both human history and biology when considering the impact of large epidemics in the more recent past, as well as the effects of infectious agents on infant mortality across time.

Biological approaches in microevolutionary studies during the second half of the 20th century were used to test hypothesis that specific genotypes may confer immunity or resistance to certain infections and that some frequent genetic conditions may be adaptive in one context and maladaptive in another. Allison (1954) was the first to propose that the heterozygous condition of sickle-cell trait, where the individual has inherited one gene for the prevalent hemoglobin from one parent and the sickling hemoglobin S from the other, was prevalent in regions of Africa with high Plasmodium falciparum malaria, the most life threatening form of malaria. Thirty years later Durham
(1983) provided the statistical correlation between malaria prevalence and sickle-cell gene frequencies in West Africa. Moreover, the classical anthropological work by Livingstone (1958) related the widespread distribution of sickle-cell trait in West Africa to the introduction of iron tools and subsequent agriculture and deforestation that increased the available breeding ground for *Anopheles gambiae*, the major mosquito vector, allowing malaria to become established among settled populations and as a selective agent for sickle-cell trait.

Other studies using a biological approach attempt to reconstruct epidemiological patterns of disease transmission among prehistoric and historic populations. The aims of this type of research are first, to establish the antiquity and evolution of infectious diseases in human populations by examining skeletal remains as well as soft tissue evidence, and secondly, to contextualize these findings to the physical and cultural circumstances of the human population involved. It is in this tradition that this thesis research is framed. Paleopathology, as an example of this approach, has had a particularly decisive role in the debates over the antiquity and origins of tuberculosis and syphilis in the Old and New World. Ethnographic analysis has also been applied for paleoepidemiological reconstructions of infectious disease patterns in prehistory (Dunn 1968, Goldstein 1969, Cockburn 1971 and Black 1975). Examining disease patterns and serological evidence in contemporary hunter-gatherers and extrapolating these findings, these scholars hypothesize that due to their small size, mobility, and relative isolation from other groups, ancient hunter-gatherers were free from the acute, epidemic infectious diseases that afflicted later agrarian societies. In early food-foraging groups, populations of no more than 200-300 individuals would not have been large enough to

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sustain acute epidemic infections like measles that would run their courses and then die out by leaving an immunized surviving population.

During the 1980's, substantial anthropological research focusing on the co-evolution of human culture and infectious disease demonstrated that cultural transition profoundly affects the nature of infectious disease patterns. The health consequences of such transitions are exemplified in the transition from food foraging to food production documented in the collection *Paleopathology at the Origins of Agriculture* (Cohen and Armelagos 1984). Contrary to the expectations, the twelve (out of eighteen) case studies across the globe that reported on the incidence of infection in this collection, found an increase in infection rates from the time of late hunter-gatherers to early farmers. The increased infection rate was the clearest major trend of the collected data. The incidence of infectious diseases was measured using the frequency of nonspecific skeletal lesions of infectious etiology as well as by the frequency of specific diseases such as tuberculosis and treponematosis. These data contributed significantly to the resolution of a long standing controversy in anthropology concerning the relative health of hunter-gatherers and farmers, suggesting that increased infection in early farmers resulted from some combination of sedentism, larger population aggregates and the well established synergism between infection and malnutrition. Cultural transitions profoundly affect the nature of infectious disease pattern.

Ecological approaches are amongst the most applied to the study of infectious diseases. Disease ecology was pioneered by the medical geographer May, who formalized the role of environment – both physical and sociocultural- in the study of infectious disease problems (May 1958). The model can be seen as an extension of the
epidemiological triad of host, pathogen, and environment and served well to demonstrate many human behavioral and environmental factors in the transmission and spread of infectious diseases. Examples of this type of research are the demonstration by May of how the higher transmission of malaria amongst migrants from lowland to highland in North Vietnam was related to the transplanted ground-level dwellings of the lowlands without modification to the highlands, exposing the newcomers to low-flying mosquito vectors. In contrast, native hill people constructed stilted homes with living quarters above the mosquito’s flight ceiling. Further work by May showed how hookworm infection rates amongst workers in China depended upon the environment in which individuals worked. Rice field workers exposed to raw human feces in the fields exhibited high infection rates as opposed to silkworm farmers who spend their days on ladders tending to mulberry leaves. During the mid 1980’s Turshen (1984) argued that the use of this model that focuses on downstream problems was the reason why scholars fail to elucidate why disease epidemics occur at certain historical moments. In other words, this model was inadequate because it fails to consider the ultimate cause of disease, which Turshen proposed are economic, social, and political. This is also the guiding paradigm of critical medical anthropologists working on the etiology of the type 2 diabetes epidemics among Native Americans, and that have shown how the roots of the epidemics lie deep within the structural inequalities engendered through conquest, colonization, and capitalist development (Benyshek et al. 2001). The political ecology of disease has largely influenced more recent disease ecology research focusing on the consequences of ecologically ill-advised development schemes, such as diseases as the
consequences of environmental disruptions caused by large-scale, internationally sponsored projects.

And finally the third type of approach is sociocultural which relates the role of human behavior in increasing or limiting infectious disease transmission. Numerous anthropologists have noticed the importance of understanding culturally prescribed and proscribed behavioral practices and their impact on infectious disease transmission. Of interest here is the fact that not every human behavior is adaptive in an evolutionary sense because many culturally prescribed patterns of behavior actually promote disease spread. Notably the role of mortuary practices among the Fore and the transmission of kuru (Steadman and Merbs 1982), the use of ‘nurse dogs’ by Turkana females of Kenya, to lick and clean children, in the transmission of echinococcosis also known as hydatid disease, and the role of religious use of water amongst Muslims of Egypt in the transmission of Schistosomiasis, to provide a few examples.

As noted by Inhorn and Brown (1990) a focus on infectious disease requires us to bridge the gap between the cultural and the biological, but the struggle between biological and social explanations of human life is nowhere more pronounced than in anthropology. Biocultural synthesis has not been a central concern of anthropological theory in the past two decades as noted by Goodman and Leatherman (2001). To address this issue and work towards a biocultural sythesis they organized a Wenner-Gren international symposium in 1992 entitled “Political-Economic Perspectives in Biological Anthropology: Building a Biocultural Synthesis”. As the authors note, sociocultural anthropologists generally have been inattentive to the biological consequences of changing cultures and environments and, biological anthropologists have not paid
attention to how large-scale political economic processes interrelate with local-level
ecologies to shape biologies. They see the need for synthetic approaches that incorporate
the diversity of knowledge and approaches in anthropology and that provide an effective
framework for analysis of how the process of inequalities and social change interact with
human biologies. The work on the "developmental origins" of cardiovascular and
metabolic disease is an excellent example of this type of synthetic approach (Benyshek
et al. 2001; Benyshek et al. 2006; Benyshek and Watson 2006; Martin et al. 2000). The
fundamental idea in this multidisciplinary approach is that early in life, mainly in the
embryonic, fetal and perhaps the postnatal period, mammals make irreversible choices in
their developmental trajectories, in a sense predicting the environment into which they
will be born or grow up, and in order to maximize their chances of reproductive success
as adults (Gluckman and Hanson 2005).

Four decades of work in search of human genetic adaptations like the sickle cell trait
– endemic malaria connection mentioned earlier, have failed to produce other instances.
Only recently HIV infection has been linked in Africa with increased frequencies of
alleles that confer disease protection. There seem to be few situations in which human
populations develop a genetic adaptation to a local and specific environmental challenge
and research reaffirms that biological plasticity is a species-wide adaptive mechanism,
making humans adept at rapid, plastic adjustments to a range of environmental
conditions (Goodman and Leatherman 2001). Developmental plasticity has been
proposed to explain why Yanomamo Indians of Brazil upon contact with outsiders and
exposure to *Mycobacterium tuberculosis*, suffered high rates of infection and disease
beyond simply assuming host naiveté (Sousa et al. 1997). Serological studies showed
that a large proportion of the population was producing a predominantly Th2 immune response, which is needed to fight extra cellular pathogens such as helminthic worms. To fight tuberculosis the Th1 response is the more successful of the two types, because it aims at intracellular organisms like *M. tuberculosis*. Although humans are capable of producing both, Th1 and Th2 responses, the two down regulate one another.

Ramenofsky, Wilbur and Stone (2003) note that disease loads have not been considered in models of disease spread in the Americas.

Infectious disease emergence in prehistoric populations has been influenced by multiple factors as noted in this brief review. Host genetics, host developmental plasticity, disease loads, cultural practices and behaviors, and political economics, all of which facilitate or limit disease spread and manifestations. Anthropological research has centered on the adaptability of humans but not so of our pathogens when studying infectious disease. The hallmark of cultural changes or transition has been the increase in infectious disease rate. Providing a timeframe for the co-evolutionary process between humans and their bacterial pathogens will be essential in understanding the process by which pathogens adapt, particularly those adaptations resulting in increased virulence and/or epidemics for the human host. Microorganisms undergo mutations in their genomes at a faster rate than humans due and are characterized by relatively small genomes, both advantageous aspects in the study of pathogens genetic adaptations in the past.
Ancient DNA Research and Human Pathogens

With the advent of molecular techniques scholars adventured into the arena of the amplification of ancient DNA (aDNA). As early as 1984 Higuchi reported the identification of DNA from a museum specimen of the extinct quagga, and showed its phylogenetic relationship to modern zebra. Anthropologists were quick to recognize the potential of molecular techniques to address questions long investigated in the field and soon followed Pääbo’s work obtaining sequence data from a 2,400-year-old Egyptian mummy (1985a,b 1986). The development of the polymerase chain reaction (PCR) by Mullis and Faloona (1987) revolutionized DNA research in general and aDNA in particular. This technique is based on the complementary nature of the DNA bases in the two strains that form the double helix. With the use of an enzyme involved in DNA replication, millions of copies can be produced of a single, specific DNA target in vitro, rendering possible the analysis of the genetic material of deceased organisms. As discussed in Pääbo (1993), PCR greatly increased the ability to reliably and reproducibly type ancient genetic markers. The application of aDNA techniques allowed, for the first time, researchers to track temporal genetic changes. There have been several reviews of aDNA methods specific to anthropological applications (Sykes 1993, Brown and Brown 1994, O’Rourke et al. 1996, O’Rourke et al. 2000, Brown an Pluciennik 2001) including the ethical, legal and social issues involved (Kaestle and Horsburgh 2002). Within anthropology, aDNA techniques permit the analysis of patterns of genetic variability in both human and nonhuman organisms, to test hypothesis of human origins, behavior and human disease. Although early and enthusiastic aDNA studies had suggested that DNA was recoverable from remains more than a million years old, later work has shown that
this was due to contamination from modern sources. It does not appear that, beyond the exception of insects trapped in amber, DNA preserves longer than 130,000 years (Stankiewics et al. 1998). A recent report from a Spanish led team working in the Atapuerca cavern complex in northern Spain (Valdiorsera et al. 2006) however, claims to have isolated mtDNA from a 400,000-year-old cave bear (*Ursus deningeri*).

Following the initial enthusiasm, the challenges facing aDNA studies became apparent, such as the difficulties generating sufficient authentic DNA sequences to make a study conclusive. The nine criteria for authenticity proposed by Cooper and Poinar (2000) are the result of a process that started with simple suggestions (e.g. using negative controls or analyze of multiple extracts per sample) evolving over time into a more detailed and extensive list of requirements (Handt et al 1994, O’Rourke et al. 2000). These include: (a) Isolation of work areas to separate samples and extracted DNA from PCR amplified products; (b) negative control extractions and amplifications to screen for contaminants entering the process at any stage; (c) appropriate molecular “behavior”, meaning the observation of an inverse correlation between amplification efficiency and size of amplification product, a reflection of the degradation and damage in the ancient DNA template as demonstrated by Pääbo (1989), [consequently the success in amplifying large DNA fragments in ancient DNA studies should be treated with caution]; (d) reproducibility where multiple PCR and extractions should yield consistent results; (e) cloning of products to assess for damage, contamination and jumping PCR; (f) independent replication with the generation of consistent results by independent research groups; (g) biochemical preservation of other biomolecules that correlate with DNA preservation (e.g. collagen or amino acid racemization) as an
indicator of sample preservation, (h) quantification by competitive or Real-Time PCR to give an indication of the number of starting templates in the reaction; and (i) associated remains equally well preserved with no evidence for contamination.

Cooper and Poinar (2000) argue that in the absence of compliance with these criteria, the reliability of results is uncertain. In an assessment of ancient DNA studies however, Gilbert et al. (2005) point at the surprisingly low number of studies that actually have applied the nine criteria. The authors note that the authenticity and reliability of ancient DNA data arise from a complex set of poorly understood areas of knowledge, mainly those of DNA damage and contamination, and as such, no clear cut answer exists as to what makes a study reliable. Many researchers view the complete application of the criteria as unreasonable and selectively adopt criteria to fit their situation, as it is widely accepted within the field that contamination affects samples differently in distinct ancient DNA studies. If we consider research conducted on ancient DNA of extinct animals such as the dodo (Shapiro et al. 2001), giant eagle (Bunce et al. 2005) and moa (Haddrath and Baker 2001), they are clearly at less risk of environmental contamination compared with studies investigating human DNA or the DNA of paleopathogens for which the modern counterpart may be ubiquitous. Gilbert et al. (2005) advocate for a more cognitive approach with regards to assessing the reliability and conclusions of aDNA data. Instead of planning or assessing studies by using criteria as a check list, consideration should be given on a case-by-case basis as to whether the evidence presented is strong enough to satisfy authenticity given the problem.

The earliest published report on the finding of bacterial DNA from ancient human bones was by Spigelman and Lemma (1993) documenting the isolation and

Through this work it has been possible to answer questions that long puzzled paleopathologists, such as proving that tuberculosis pre-existed European contact in Asia and the New World and that *M. tuberculosis* and not *M. bovis* was the prevalent etiology of tuberculosis in past human populations.

Other human pathogens successfully detected in ancient human remains include bacteria, parasites and viruses. Instances of identification of bacteria are: *Mycobacterium leprae*, the etiologic agent of leprosy, in bone samples with morphological evidence of leprosy and dating between the 1st and 15th century A.D. (Haas et al. 2000, Taylor et al. 2000); *Yersinia pestis* the etiologic agent of plague, in dental pulp from remains of medieval Europe (Drancourt et al. 1998, Raoult et al. 2000, Drancourt and Raoult 2002); *Treponema pallidum subsp. pallidum* the etiologic agent of venereal syphilis in the femur of an Easter Island human remain dated 200 years old (Kolman et al. 1999), and notably the only one that succeeded so far. Cases of identification of parasites with aDNA methods are *Plasmodium falciparum* one of the etiologic agents of malaria.
identified in Egyptian mummified tissue and Roman bone remains (Taylor et al. 1997, Kolman et al. 2000, Abbot 2001), and Trypanosoma cruzi the etiologic agent of Chagas disease identified in bone and mummified tissues of the Andean region (Guhl et al. 1997, Ferreira et al. 2000, Madden et al. 2001, Aufderheide et al. 2004). Two viruses have been identified so far, the T-cell lymphotropic virus type I (HTLV-I) in mummified and skeletal material from the Andean region (Li et al. 1999, Sonada et al. 2000) and the 1918 Spanish Influenza pandemic virus that killed an estimate of 20 to 50 million people worldwide. Material was obtained from an Alaskan influenza victim who was buried in permafrost in November 1918 and from archived formalin-fixed lung autopsy materials (Taubenberger et al. 1997, Taubenberger et al. 2000). Notably, the complete sequences of the eight viral RNA fragments have been obtained for the 1918 virus, a process that took ten years to complete due to the fragmentary nature of ancient nucleic acids. With the use of reverse genetics it has been possible to revive the virus to study the properties associated with its extraordinary virulence (Tumpey et al. 2005) and phylogenetic studies place it in the group of avian influenza viruses that adapted to humans in toto as opposed to human/avian reassortant viruses of the 1957 and 1968 pandemics (Taubenberger et al. 2005). This is an unprecedented example in which the study of nucleic acids of an ancient pathogen has shed light on the origins of a pandemic human disease and is contributing to understanding the mechanisms of emergence of virulence of the pathogen.

All reported ancient pathogen research described above relied upon the use of species-specific PCR primers. In two instances researchers have used universal bacterial primers targeting the 16s rRNA gene of bacteria to successfully amplify bacterial DNA.
of the colon of an Andean mummy (Ubaldi et al. 1998) and the colon and stomach of the Tyrolean Iceman Ötzi (Cano et al. 2000). There is increasing evidence that the bone marrow is a source for the preservation of bacterial pathogens that developed bacteremic disease before death, as in the case of miliary (generalized) tuberculosis which has yielded positive findings of MTBC sequences in unremarkable ancient bone. Spigelman and Donoghue (2003) have found that M. tuberculosis DNA is some times more abundant than the host’s mitochondrial DNA when analyzed under identical conditions. Some of the explanations advanced are the preferential preservation of DNA from prokaryotes due to the mechanisms of DNA protection that exist in prokaryotic cells. There is evidence that bacterial DNA preserves better in bone than in comparable soft tissue (Hagelberg and Clegg 1991, Lassen et al. 1994), and given this, a reason for which bone was thought to be a good possible source of ancient microbial DNA in this thesis research.

Molecular Taphonomy and Bone Diageneisis

After death the repair mechanisms that maintain DNA integrity cease and cellular compartments disintegrate allowing for the release of catabolic enzymes with the consequent DNA degradation. For an excellent review on DNA damage processes see Pääbo et al. 2004. The predominant damage in ancient samples is the degradation of DNA to small fragments generally between 100-500 bp. This reduction is caused by both enzymatic processes post mortem and nonenzymatic hydrolytic cleavage of phosphodiester bonds in the phosphate-sugar backbone that generates single stranded nicks. Hydrolitic cleavages also occur on the glycosidic bonds between the nitrogenous
bases and the sugar resulting in abasic sites. During aDNA amplifications and cloning the common missincorporations are C to T and G to A, both considered a consequence of base deamination. Another type of DNA damage is caused by the crosslink of DNA with other biomolecules and oxidative lesions to the bases and deoxyribose residues. Under rare circumstances though, DNA may escape hydrolytic and microbial degradation, such as when it becomes rapidly desiccated or when it absorbs to a mineral matrix.

The biomedical, forensic, and anthropological literature is sparse in regard to systematic studies of bone marrow changes in the post mortem interval and later taphonomic stages. Forensic scientists have determined that the morphology and cytochemistry of haematopoietic cells in the bone marrow of cadavers is not helpful in determining the post mortem interval after 12 hours of death, when bone marrow cells are all undergoing autolysis (Findlay 1977). A common statement found in the literature (Spigelman and Donoghue 2003) is the occurrence of peri-mortem bacteremia with organisms from the intestinal flora spreading all over the human body. Surprisingly, no systematic study is sited or can be found regarding this issue. Contrary to this general statement, one systematic study performed on a mummified individual to assess bacterial distribution in different organs found that only the intestine is rich in bacterial DNA. Other compartments such as liver, lungs and the diaphragm produced no signal or very week signal (Rollo and Morata 1999).

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unremarkable ancient bone (Faerman et al. 1997, Fusegawa et al. 2003), Spigelman and Donoghue (2003) have found that *M. tuberculosis* DNA is some times more abundant than the host’s mitochondrial DNA when analyzed under identical conditions. Some of the explanations advanced are the preferential preservation of DNA from prokaryotes due to the mechanisms of DNA protection that exist in prokaryotic cells. There is evidence that bacterial DNA preserves better in bone than in comparable soft tissue (Hagelberg and Clegg 1991, Lassen et al. 1994), a reason why bone may be a good source of ancient microbial DNA -- as addressed in this thesis research.

Paleopathology of Infectious Disease in northern Chile

Archaic coastal populations suffered from fish tapeworm infestation as identified by ova of *Diphillothrium pacificum* in coprolites (dried feces) of a significant number of individuals (Reinhard and Aufderheide 1990). Populations in the valleys with an agro­pastoral subsistence of later periods also consumed fish but exhibit no evidence of fish tapeworm (Aufderheide et al. 2002), and this fact is seen as evidence of consumed fish being cooked by these later people.

Chagas disease, caused by the protozoan parasite *Trepanosoma cruzi* is transmitted by the contact with feces of ruduviid bugs of the family Triatominae. The most prevalent species locally known as *vinchuca* is *Triatoma infestans* found frequently in the roofs and walls of rural houses. Rothammer et al.(1985) reported nine possible cases of Chagas’ disease in mummies from northern Chile dating between 2,450 -1,400 B.P. based on the presence of “megavisceral syndromes” such as enlargement of the colon (megacolon), esophagus (Achalasia) and heart (Chagas’ cardiopathy). More recently,
biochemical methods including aDNA have confirmed a record of the disease in the region for the past 9,000 years (Aufderheide et al. 2004). The acute phase of the disease is characterized by a transient “chagoma” or inflammation of the eye close to the insect bite area followed by a prolonged asymptomatic period up to several decades. Late manifestations include cardiopathy, achalasia of the esophagus and megacolon. Aufderheide et al. (2004) found a disease prevalence of 41%, a very high rate indeed.

Standen and Arriaza (2000) reported skeletal evidence attributable to a chronic infection, with a pattern identified as Yaws type non-venereous treponematosis, in archaeological remains from the Atacama Desert in northern Chile. In the study, 8% (n=51) of a total of 636 examined individuals were affected. The period investigated a span of over 4,000 years, between 5,000 and 800 B.P. The incidence was significantly higher in coastal fishing and hunting-gathering populations, reaching 18.5% (33/178), in comparison with the valley agriculturalist populations where it reached only 3.9%. There was no difference in disease incidence for both sexes, and in respect to age distribution, adults exhibited a higher (88.2%) incidence compared to sub adults (11.8%). Clearly the highest incidence is observed among arcahic coastal populations—reaching a peak of 20%--with a decline in later periods. In the valleys there is a slight increase over time from 2.3% during the formative period to 6.3 % in pre-contact populations. The authors attribute the different incidence rate between the coast and valleys to the very different ways of life and clothing habits of coastal and valley populations. Yaws is transmitted through skin lesions during childhood and the people at the coast covered only their genital region as opposed to the people in the valleys that used ‘camisas’ that covered trunk and upper limbs, thus providing a barrier for transmission. Standen and Arriaza
(2000) have advanced another hypothesis to explain the higher incidence of treponematosis among archaic coastal populations, namely their mortuary practices. Archaic coastal populations belong to the Chinchorro culture that practiced artificial mummification involving extensive manipulation of corpses including the complete defleshing of bones. This mortuary practice was discontinued by 3,700 B.P at the beginning of the formative period.

Tuberculosis has also been reported for the region and confirmed with aDNA methods (Arriaza 1995). Interestingly it appears in the archaeological record after the adoption of an-agro pastoral subsistence (Allison 1981). The incidence for skeletal tuberculosis is estimated to have reached 10%.

Evidence from preserved soft tissues in mummies shows that pneumonia was the major cause of death in individuals from both coast and valley populations across all time periods, independent of diet or social organization. Nearly 69.9% of the 51 mummies examined exhibit bilateral pneumonia that could be clearly identified as bronchopneumonia or lobar pneumonia (Allison 1984). Microscopic examination of affected lung tissue demonstrates that in most cases there are abundant exudates and bacteria present. Moreover, Allison (1984) reports that 44% of the mummies exhibit pleural adhesions, which suggest that nearly half of the examined individuals had had at least one previous episode of pneumonia. The very high incidence of pulmonary infections in the region is remarkable. For a period that extends back over 7,000 years, pneumonia has been observed in all its major variants in infants and adults, including all forms of complications such as lung abscess, pleurisy, pericarditis and endocarditis.
Surprisingly however, no further studies have attempted to determine what species of bacteria are the etiologic agents of pneumonia in these populations.

The void of knowledge and explanatory models for this astonishing incidence of bacterial disease that do not leave traces in the skeleton was one of the strong motivations for the current study: the development of a methodology that would allow us to establish the etiologic agents that were active in these populations, and thereby further clarify the epidemiology of the region.
CHAPTER 3

MATERIALS AND METHODS

Populations Under Study

“The arid conditions that shaped their lives also shaped them in death.”

Allison 1984

Many scholars propose a maritime foundation for the Andean civilization (Raymond 1981, Llagostera Martinez 1979). The earliest sites in the South Central Andes are located at the coast and date back to 11,000-12,000 years B.P. (Keefer at al. 1998). Archaeological evidence demonstrates that these groups of people were able to exploit the sea with remarkable effectiveness, enabling the establishment of large sedentary populations earlier on the desert coast of the South Central Andes than in any other part of the New World. The earliest sedentary populations appear around 9,000 B.P. (Benfer 1984, Quilter 1989) and population estimates by 1520 A.D. range between 6 million (Rowe 1947, Smith 1970) and 13 million (Cook 1981). The continuous human occupation over a period of 9,000 years, the excellent skeletal preservation provided by the desert environment, and additionally, the artificial mummification practiced by the Chinchorro people living at the coast of Northern Chile and Southern Perú between 7,000 to 3,100 B.P., have yielded close to 2,000 human remains kept at the Museo Arqueológico San Miguel de Azapa in Arica, Chile.
The three populations considered for the purpose of this study are Morro -1/6, Azapa-140, and Lluta -54 (see map). The first two populations inhabited the Azapa Valley, Morro 1/6 at the Pacific coast were hunter-gatherers with a maritime subsistence during the late archaic period (2,500 - 1,700 B.C.) and Azapa-140 were agriculturalists who occupied the site during the Early Intermediate period (B.C. 1700 – A.D. 500) and the Middle Horizon (A.D. 400-1000). The third population is from the neighboring Valley of Lluta and located 60 km inland up the mountains corresponding to a late pre-contact period occupied by a population with an agro-pastoral subsistence. The Azapa and Lluta Valleys are located in the south central Andean region and are part of the occidental valleys (Lumbreras 1981) that characterize the pacific Andean slope of southern Peru and northern Chile. They are formed as the result of the action of river.
streams originating in the western slope of the Andes. The existence of these rivers allowed human settlement in the region. The Lluta and Azapa valleys are both part of the Atacama Desert with extreme daily temperature oscillations and scarce rainfall (Sepúlveda 1962, Villagrán 1982). The Pacific Ocean with its cold water is rich in salt and nutrients. A variety of fishes (anchoveta, jurel, tuna, albacora, etc.), crustaceans, sea mammals (e.g. sea lion – *Otaria florecens*-, and chungungus – *Lutraferina peruvensis*), rats, chillas (*Seudalopex griseus*), insects (e.g. spiders and scorpions) and birds (e.g. pelican – *Pelecans thagus* -, and piqueros – *Sula dactylatra*), are some of the resources available in this area (Quintanilla 1983).

The San José River originally formed the Azapa Valley through erosion and the Lluta River formed the Lluta Valley by the same mechanism. The water of these rivers had been used in agricultural practices since prehistoric times. Some of the cultivated products are *yucca*, pumpkin, chili, tomato, cotton, avocado, and corn. Some of the native trees in these valleys are chañar, (*Geofrea decorticans*), algarrobo (*Prosopis atacamensis*) and pimiento (*Schinus molle*; Quintanilla 1983). Faunal resources here include snakes (e.g. *Dromicus angustilineatus*), lizards (*Phylodacylus inaequaliscope* and *P. heterurus*), batrachianas (*Bufo atacamensis* and *B. kalinowskii*), and birds (e.g. *Zontrichia capensis antofagastae*).

Materials

Bioarchaeological Samples: For the purposes of this study, I obtained bone marrow samples of 30 well preserved, partially mummified individuals from three archaeological sites (10 samples per site) spanning between 600 and 4,500 years B.P: Morro (Mo-1-6),
Azapa (AZ-140) and Lluta (LLU-54) (see site descriptions above). For each site, 10 adult skeletal remains unremarkable for infectious diseases were selected and bone marrow was extracted during a one-month field season. The bone surfaces were scraped with sterilized sand paper. The bone marrow samples of ~100 mg were obtained aseptically with disposable Jamshidi bone marrow biopsy needles from the posterior superior iliac spine and stored in DNAse free micro-centrifuge tubes at constant room temperature (25°C) until the analysis was performed. The samples were transported to the United States with accompanying authorization letter and permission for destructive analysis obtained at the Direction of the Museo Arqueológico San Miguel de Azapa.

Methods

DNA extractions of bone marrow samples were performed in the ancient DNA laboratory of the University of Utah where I received training on ancient DNA methods and worked under the supervision of Dr. Dennis O’Rourke. For contamination control all ancient DNA procedures were conducted in two physically separate ancient DNA laboratories, one devoted to DNA extractions and another room for post PCR procedures. The PCR cylinder itself was located in a third room. These laboratories are cleaned with a 30% bleach solution and all working surfaces are subjected to Ultra Violet light to cross-link contaminating DNA. Clean laboratory coats, gloves, face masks, head covers (hoods) and arm covers are put on at the entrance of the ancient DNA laboratory. All reagents and sterile water are purchased as molecular grade, DNA- and DNase/RNase-free directly from the manufacturer and are tested for exogenous DNA contamination regularly. Two different methods of extraction were applied (a)
Proteinase K buffer and PTB followed by silica based cleaning methods and (b) L6 Buffer with proper controls (see appendix I for experimental protocols).

The degree of DNA preservation was established using polymerase chain reaction (PCR) amplification of human mitochondrial DNA (mtDNA). PCR procedures were done in individually capped PCR tubes to insure that each tube is covered when not in use. PCR cocktails were made in 25 µl per sample containing manufacturer’s buffer, 20 µM primers, 1.5 mM MgCl₂ Deep Vent Polymerase, 200mM dNTPs and sterile water to bring up volume to 25 µl per sample. The PCR cycling involved 5 minutes denaturation at 95 °C followed by 40 cycles of denaturation at 95 °C 1 minute, annealing at 58 °C for 1 minute and extension at 72 °C for 30 seconds. For DNA visualization 10 µl of PCR product along with 2 µl loading dye were loaded onto a 2% agarose gel containing ethidium bromide. The gel images were photographed using Polaroid camera on a UV transilluminator. The samples that were positive for mtDNA amplification were then further assessed for the presence of bacterial DNA using universal bacterial primers (Greisen et al. 1994) to amplify a 229 base pair fragment of the 16S rDNA gene. Each bacterial species exhibits a unique and diagnostic sequence of the 16S rDNA gene as shown by the work of Carl Woese during the 1970’s who, by comparison of ribosomal RNA (rRNA) sequences of this gene was able to establish a molecular sequence-based phylogenetic tree, which he used to relate all organisms and reconstruct a history of life on Earth (Woese and Fox 1977, Woese 1987). Not all human pathogens, however, can be distinguished at the species level using this gene, as is the case of the different treponema and the Mycobacterium tuberculosis complex. The goal of this study however, was to search for pathogens other than treponema and mycobacteria. Positive
samples defined as a visible band of desirable bp-length on gel-electrophoresis were
subjected to automated sequencing using the Big Dye Terminator Cycle Sequencing
Ready Reaction Kit v3.0 (Applied Biosystems) visualized through an ABI 377XL
automatic sequencer. The obtained sequences were compared to gene bank sequences at
the National Center of Biotechnology Information NCBI using the BLAST algorithm to
determine the species of bacteria present in the bone marrow sample.
CHAPTER 4

RESULTS

During the process of extraction of bone marrow samples I noticed significant differences in the state of preservation for the three sites. For the oldest samples (Mo 1/6), the bone exhibited high porosity and it was difficult to obtain sufficient material for analysis, consisting mainly of powder. The most recent samples (LLU 54), in contrast, consisted of complete bone marrow cylinders with dark material filling the trabecular spaces.

DNA extracted from the 30 bone marrow samples was evaluated for DNA preservation using established human mitochondrial DNA primers. Eight of the 30 samples yielded a band of expected size in gel electrophoresis. These samples’ provenience was from the sites of LLu 54 and AZ 140. None of the Mo 1/6 samples yielded any amplifiable mtDNA. Only samples with demonstrable preserved human mtDNA were further analyzed for the presence of bacterial DNA. The first attempt amplifying bacterial DNA with universal primers of these eight samples gave a visible band of expected size in all amplifications, including blanks. It has been reported in the medical literature that the enzyme polymerase can be a source of false positive results due to the fact that the enzyme has been produced in Escherichia coli. Although the enzyme has been purified it is not possible to eliminate trace bacterial DNA. In order to overcome this problem I used and additional ultrafiltration step as well as pretreatment
of the reaction mix with DNAse before adding primers and template. With these additional steps I obtained bacterial DNA bands in three of the samples, two from LLU 54 and one from AZ 140. The sequences were identified as *Staphylococcus epidermidis*, *Pseudomona aeruginosa* and *Bacillus sp*, none of which are likely ancient human pathogens.
CHAPTER 5

DISCUSSION AND RECOMMENDATIONS

The primary interest of this study was to develop a methodology to study ancient bacterial disease that does not primarily affect the skeleton. The use of universal bacterial primers for the detection of ancient pathogens proved not to be an optimal method in epidemiological studies of ancient bacterial disease for several reasons.

1. The first problem during the research was the impossibility of generating a primer set that would specifically amplify bacterial DNA and at the same time have an optimal fragment length below 200 bp. Although multiple instances of ancient DNA research have reported the amplification of 500 to 1000 bp fragments, shorter fragment sizes are more likely to yield aDNA amplifications. Unfortunately, any amplification fragment of the 16s rDNA gene shorter than 229 bp would concomitantly amplify human DNA, which is certainly more abundant in the bone marrow samples used in this research.

2. Another problem that emerged during this investigation was the presence of *Escherichia coli* DNA that comes with the commercially obtained enzyme DNA polymerase required for DNA amplification by means of PCR. Biotechnology companies produce this enzyme in laboratory strains of *E. coli*, and although the enzyme is purified, genomic bacterial DNA is known to be present in small amounts. In order to still be able to use universal bacterial primers, the condition was set that any *E. coli* DNA found during the research had to be eliminated, as we could not distinguish if it
had come with the enzyme reagent or if it was present in the bone marrow sample being studied. In order to further minimize the amplification of residual *E. coli* DNA, I opted for adding a DNAse digestion step of the master mix before the addition of the primers and template DNA. This additional step was not sufficient and I added an ultracentrifugation step after the addition of the primers. It was only when both additional steps were used that reproducibly blanks no longer showed amplification bands at 40 cycles.

3. When analyzing the sequences obtained, it became clear that the method was ideal to amplify contamination. *Pseudomonas* and *Bacillus* are both soil bacteria and could have contaminated the sample during collection or in the laboratory. Both have been described in the medical literature as agents of invasive human disease. These reports, however, have been restricted to hospital transmission and intravenous drug abuse cases, both of which are circumstances unlikely to have contributed to disease transmission factors in the studied populations. The sequences of *Pseudomonas* and *Bacillus* found in this study both exhibit several mismatches which were not predominantly of the missincorporation type C to T and G to A described earlier. Although the damage of DNA can not be excluded as the reason for the difference in sequence with the gene bank data, it is also possible that we are in the presence of so far not described bacterial sequences. *Staphylococcus epidermidis* is a commensal of the human skin and could have reached the sample during collection or during laboratory work. Cloning and sequencing of the amplified DNA fragments was not contemplated in this study as none of the laboratories involved had available resources for this purpose at the time the
laboratory work was conducted. Cloning would increase the chances of detecting a template present at lower copy number than a contaminant.

Although the development of a methodology that would allow for the study of bacterial pathogens at the population level from skeletal remains is desirable, in order to first establish disease etiology, I suggest future work aims at the detection of bacterial pathogens in soft tissue with lesion evidence. To reach this aim I propose the use of a combined approach using universal bacterial primers followed by cloning and sequencing as well as the use of specific primers aiming at determining bacterial pathogens for which a long evolutionary history is likely. For example the geographically patterned serotype distribution of *Streptococcus pneumoniae* and *Haemophylus influenza*, both major etiologic agents of contemporary pneumonia worldwide, makes them good candidates for a long co-evolutionary history. The remarkable high pneumonia rate in Andean prehistoric populations warrants the specific search for these pathogens to expand our knowledge of their disease antiquity.

The bone marrow poses another problem as a source of bacterial pathogens. The distribution of the pathogens may be uneven and prevent reproducibility of the results. In the early phase of determining disease antiquity of paleoepidemiological research, it seems more prudent to obtain samples from mummified lung tissue exhibiting clear evidence of acute bacterial infection.

The continuous innovations and improvements in molecular methods and bioinformatics have yielded several promising avenues to study ancient pathogens as well as human evolution. The development of whole genome shotgun sequencing has allowed the discovery of a great number of microorganisms that can not currently be
cultured, posing similar limitations as does aDNA pathogen research. Recently, Svante Pääbo reported in the Biology of Genomes meeting at New York’s Cold Spring Harbor Laboratory that they had managed to sequence around a million base pairs of Neanderthal nuclear DNA extracted from a 45,000 year old male specimen from Vindija Cave in Croatia using this method (Dalton 2006). The sequencing method was developed by 454 Life Sciences in Branford, Connecticut, and allows genetic fragments of 100-200 base pairs in an emulsion to be sequenced directly in tiny wells. The fragmentary nature of ancient DNA is not a limitation for this method. It has been used to obtain whole genomes of many modern human pathogens and it will certainly aid in future research addressing emergence and evolution of ancient pathogens by means of comparative genomics.

The significance of this work resides in the efforts at developing a methodology to study co-evolutionary time scale between humans and their bacterial pathogens. The evolutionary perspectives addressing host-pathogen interactions at the molecular level and across time are the most promising strategies to learn about virulence and disease emergence as a result of changes in human behavior. The South Central Andes represent an ideal research area across time, with unprecedented well-preserved remains, extending back in time continuously for more than 10,000 years and awaiting to contribute in our understanding of infectious diseases emergence. As medical anthropologists, understanding how pathogens have become a threat to human health by exploring host-pathogen co-evolution, we are offering a means to this end.
## APPENDIX I

### SAMPLES ANALYZED

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<th>Site Azapa 140</th>
<th>Site Lluta 54</th>
<th>Site Morro 1/6</th>
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<td>M1/6 – T19C1</td>
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APPENDIX II

EXPERIMENTAL PROTOCOL

DNA extraction using Proteinase K and PTB

Proteinase K Buffer

50mM Tris pH 8

450 mM EDTA pH 8.5

0.5 % tween 20

1. Proteinase K digest: Proteinase K is added to 100ml Proteinase K buffer and aliquoted in 1.7 microcentrifuge tubes (MCT).

2. 1.2 ml of Proteinase K digest and 120 µl of 0.1 M PTB in 10mM sodium phosphate buffer are added to 100mg of bone marrow sample and incubated overnight (18h) at 56 °C shaking.

3. The digests are transferred to Centricon-30 spin columns and centrifuged at 2,000 rpm for 2.5 hours at 25 °C. A brief spin with inverted columns recovers the extract on the cup.

4. 300 µl of TE buffer are added to each cup transferring 125 µl to new 1.7 ml MCT.

5. 625 µl of PB buffer are added to each tube and the 800µl are transferred to a QIAquick column and centrifuged for 60 seconds discarding flow through.

6. 750 µl Buffer PE are added to the column and centrifuged for 60 seconds discarding flow through.
7. Elution with 40 µl of TE-Buffer into 1.7 MCT and frozen at -20°C until further analysis.

**DNA extraction using L6 Buffer**

L6 Buffer:
1. Dissolve 24 g of GuScn in 20 ml 0.1 M Tris-HCl pH 6.4, heating at 60-65 °C water bath
2. Add 4.4 ml of 0.2 M EDTA pH 8
3. Add 0.5 ml 100x Triton, mix well
4. Add 1.5 g of silica to bind any contaminant DNA, mix and centrifuge at 3000g for 15 minutes.
5. Aliquot in 1.7 ml MCT and keep at room temperature in the dark. Stable for 3 weeks.

_Extraction:_
1. Add 1000 µl L6 Buffer to 100 mg bone marrow and incubate overnight at 60 °C.
2. Transfer 250 µl to a new tube containing 750 µl NaI and 5 µl Glassmilk. Mix and incubate at room temperature (RT) for 5 minutes mixing every minute to insure glassmilk stays suspended.
3. Centrifuge at 13,000 rpm for 5 minutes and discard supernatant
4. Wash pellet with 500 µl new wash
5. Centrifuge at 13,000 rpm for 5 minutes
6. Repeat wash 2 times
7. Dry pellet at RT and resuspend in 70 µl dH₂O, incubate at RT for 10 minutes
8. Centrifuge at 12,000 rpm for 5 minutes and transfer supernatant to new tube
APPENDIX III

SEQUENCE ALIGNEMENTS

Bacillus sp.

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Pseudomona aeruginosa

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</tr>
</thead>
<tbody>
<tr>
<td>Sbjct 1181</td>
<td>TACGACCAGGGCTACACACGTGCTACAATGG-TGGTACAAGGTTGCCAAGCCGCGAG 1239</td>
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<tr>
<td>Query 72</td>
<td>GTGGAGCTAATCCCATAAACCCGTATCGTAGGTCCGGATCGCAGTCTGCAACTCGCCTGGG 131</td>
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<tr>
<td>Sbjct 1240</td>
<td>GTGGAGCTAATCCCATAAACCG-ATCGTAGGTCCGGATCGCAGTCTGCAACTCGAATGCG 1298</td>
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<tr>
<td>Query 132</td>
<td>TGAAGTCGGAATCGCTAGTAATCGTAGTGAATACGTTCCCGGGGC 190</td>
</tr>
<tr>
<td>Sbjct 1299</td>
<td>TGAAGTCGGAATCGCTAGTAATCGTAGTGAATACGTTCCCGGGGC 1357</td>
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Staphylococcus epidermidis

Query 13  TTATGATTTGGCTACACACGTCAATACTACAATACAAATACAAAGGGTAGCGAAACCGCGAG 72
Sbjct 1185  TTATGATTTGGCTACACACGTCAATACTACAATACAAATACAAAGGGTAGCGAAACCGCGAG 1244

Query 73  GTCAAGCAAATCCCATAAAGTTTTCTCTGATTGGATGTCTGCAACTGACTATAT 132
Sbjct 1245  GTCAAGCAAATCCCATAAAGTTTTCTCTGATTGGATGTCTGCAACTGACTATAT 1304

Query 133  GAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGG 189
Sbjct 1305  GAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGG 1361
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Committee Member, George L. Urioste, Ph.D.
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