PAST HUMAN HEALTH AND MIGRATION: THE ANALYSIS OF MICROBIAL DNA ASSOCIATED WITH HUMAN REMAINS RECOVERED FROM A GLACIER IN CANADA

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ABSTRACT

In paleopathology, the assessment of disease occurs through macroscopic observation, which is dependent on the preservation of the sample and the experience of the observer. Many disease events do not leave any visible signatures and therefore go undetected. The relatively new field of paleomicrobiology incorporates molecular techniques where microbial DNA, if present, is amplified from an archaeological sample. The identification of genetic material from micro-organisms, including bacteria and viruses, can confirm a diagnosis that was originally based on visible osteological or mummified tissue changes. Even more promising is the capability of molecular technology to detect microbial DNA evidence of disease processes that were not visibly evident.

Based on phylogenetic analyses of modern isolates, scientists have concluded that micro-organisms such as *Mycobacterium tuberculosis* and *Helicobacter pylori* have been associated with humans for thousands of years. *M. tuberculosis* is the causative agent of the disease tuberculosis, and *H. pylori* is known for its role in gastritis and peptic ulcers. Both are pathogenic bacteria that still impact the health of modern populations. Through the analysis of microbial DNA from these two bacteria in skeletal and mummified tissue, data can be accumulated regarding the spatial and temporal impact of these infections. Interestingly, due to the lengthy association between these bacteria and humans, phylogenetic studies on modern strains have shown that strain characterizations of both *M. tuberculosis* and *H. pylori* bacteria reveal connections with past human migrations.

In 1999, human remains were discovered eroding out of a glacier in northern British Columbia, Canada on the traditional territory of the Champagne and Aishihik First Nations. The Aboriginal elders named the site Kwäday Dän Ts'ìnchi, which means 'long ago person

found.' Radiocarbon testing of bone collagen and artifacts from the site suggested a time-frame of approximately AD 1670 to 1850, which is either pre-European contact or early post-contact for that area. I analyzed the tissues of the ancient individual specifically for genetic evidence of *M. tuberculosis* and *H. pylori* to identify partial health status and determine if a connection could be made to strains associated with European populations to clarify whether the site was pre or post-European contact.

Through polymerase chain reaction (PCR) testing of the individual's tissues with primers specific for the IS*6100* insertion sequence, TbD1, and *Rv3479*, *katG* and *gyrB* genes, I identified evidence of a possible latent tuberculosis infection. Genetic characterization of the *katG* gene associated with the ancient *M. tuberculosis* strain revealed a potential connection with European strains. Amplification and sequencing of the *gyrB* gene fragment indicated the presence of two alleles that may have been the result of a selective pressure.

PCR testing of the individual's stomach tissue with specific primers for regions with the *vacA* gene resulted in a positive identification of *H. pylori* DNA. Genetic characterization of this virulence-associated gene indicated that the strain contained a *vacA* signal (s) region s2 allele. This allele is more commonly identified in Western strains that do not cause disease, which suggests that the individual had no gastric symptoms and that European strains were present in northwestern Canada at that time. The *vacA* middle (m) region contained a hybrid m2a/m1d sequence. Modern hybrids are rare but they have been identified in Asian strains. Studies have shown that the m2a allele is more common in Western strains. A phylogenetic analysis identified that the m1d region clusters with previously published novel strains associated with Aboriginal individuals that are closely

related to Asian strains. This indicates a past connection between the ancient individual and his ancestors who arrived in the New World from Asia thousands of years ago.

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CHAPTER ONE: Introduction

As humans become more globalized, we are facing increasing challenges in meeting the needs of those who are affected by infectious diseases. The causative agents, including bacteria and viruses, are continuing to evolve, and their transmission is gaining speed due to the mobility of today's populations. The study of past disease is very important because of the knowledge that can be accumulated regarding the temporal and geographical spread of the infection. Many paleopathological diagnoses are the result of a visual assessment of skeletal material and mummified tissue. Potential evidence of an infectious disease is often limited to nonspecific tissue alterations whereby the analyst can only make an assumption about the processes involved.

Recently, the new field of paleomicrobiology has developed whereby molecular techniques are used to identify the genetic material associated with ancient tissue that may have been involved in a disease process. Only a small percentage of individuals that suffered from an infection will have any skeletal evidence, and soft tissue is normally not preserved unless natural or artificial circumstances led to mummification. Researchers often limit themselves to working with only macroscopically altered tissue due to financial and time constraints. This results in a loss of potential data when researchers focus only on abnormal tissue.

1.1 The identification of ancient infectious disease

Through the molecular analysis of ancient infections, the epidemiological information can be reconstructed and applied to current situations that may assist in preventing a new spread (Drancourt and Raoult 2005). Paleomicrobiologists study the microbial DNA from ancient remains to determine the temporal and geographical distribution of an infection. Evidence of the genetic evolution of the micro-organism itself is beneficial to the analysis of modern infections and their impact on different communities (Drancourt and Raoult 2005).

The genetic material from bacteria such as *Mycobacterium tuberculosis* has been well-identified in ancient tissues (Spigelman et al. 1993, Salo et al. 1994, Donoghue et al. 2004, Zink et al. 2007). Researchers have suggested that there is a higher chance of successfully identifying genetic evidence of this particular bacterium due to the micro-organism's hydrophobic cell wall that may protect the microbial DNA from hydrolytic and oxidative damage (Donoghue and Spigelman 2006). When an ancient tuberculosis infection is identified, further genotyping is necessary to retrieve paleoepidemiological information before the impact of the infection on a particular past population can be fully understood.

A second modern pathogenic bacterium is *Helicobacter pylori*. It is a bacterium that currently infects one in two people world-wide and has been associated with gastritis, peptic ulcers and gastric cancer (Carroll et al. 2004). Phylogenetic analyses of modern *H. pylori* isolates suggest that this bacterium has been associated with humans for millennia (Falush et al. 2003). The first direct evidence of an ancient *H. pylori* infection was recently published by Castillo-Rojas et al. (2008) with their identification of *H. pylori* DNA in the

tissues of a mummified individual recovered from a pre-European contact burial cave in Mexico. This study was important for the recognition of the antiquity of *H. pylori*, but detailed strain characterization was not included in the published research.

1.2 Microbes and ancient human migration

Interestingly, due to the long-standing association between certain microbes such as *M. tuberculosis* and *H. pylori* and their human hosts, researchers are identifying connections between the microbial strains and locations of ancient human populations. The genetic differences of the micro-organisms can be extrapolated to indicate human population differences. This information has been used to identify ancient human migration patterns (Falush et al. 2003, Gagneux et al. 2006).

For decades, theories of how the New World was first populated have been based solely on archaeological data from the analysis of artifacts and sites. Radiocarbon dating of cultural and biological materials has been used to determine possible migratory routes of the first inhabitants. Investigators originally thought that the Clovis culture was associated with the first inhabitants of the Americas. The radiocarbon dates identified with this culture indicate a short time-frame between the years of approximately 13.2-13.1 to 12.9-12.8 k calendar years (Gilbert et al. 2008). The geographical area that encompasses the Clovis sites is broad and includes most of the modern United States. Recently, archaeological information has been recovered from pre-Clovis sites, which suggests that the first humans reached the New World from Asia initially using a coastal route (Goebel et al. 2008). Scientists have analyzed mitochondrial, nuclear and Y chromosome data to

estimate that the first inhabitants arrived approximately 15,000 years ago (Goebel et al. 2008).

The study of New World human migration has included the partial and complete skeletal analyses of 'Paleoamericans' including the skeletal remains recovered from Kennewick, Washington and Spirit Cave, Nevada. Researchers were puzzled by the apparent cranial differences between these ancient individuals and modern aboriginal individuals. Multiple migrations from different geographical locations were suggested as an explanation for the cranial differences. Contrary to this, genetic data supports the idea that all aboriginal individuals share a common ancestral gene pool from a past central Asian population (Schroeder et al. 2007, Schroeder et al. 2009). Researchers have suggested that the cranial morphology differences are likely the result of genetic drift (Goebel et al. 2008).

The analysis of ancient DNA is a relatively new method that has been used to estimate the timing of human migration into the New World. Gilbert et al. (2008) identified mitochondrial DNA (mtDNA) in human coprolites with a radiocarbon date of 12,300 BP. The coprolites were recovered from Paisley 5 Mile Point Caves in south-central Oregon, and the investigators established that the mtDNA haplogroups of the samples were A2 and B2 (Gilbert et al. 2008). These haplogroups have been previously associated with the Native American founding populations (Torroni et al. 1993). The identification of authentic human mtDNA in coprolites with radiocarbon dates greater than one thousand years from the accepted Clovis culture dates is evidence in support of the theory that New World populations were present prior to the Clovis culture.

The analysis of ancient microbial DNA is also relevant to the study of New World human migration. Investigators have used modern *H. pylori* genetic data to confirm the connection between New World aboriginal populations and Asian populations (Yamaoka et al. 2002). While the modern data reveals this interesting connection, the analysis of ancient genetic material is more relevant and exciting because of the direct association with ancient humans.

1.3 The microbial DNA analysis of the tissues associated with Kwäday Dän Ts'ìnchi

On August 4th, 1999, three hunters discovered the frozen remains of an ancient individual eroding out of a glacier at 1600m in elevation in Tatshenshini-Alsek Park, British Columbia, Canada (Beattie et al. 2000) (Figure 1.1). The site is at the south end of the St. Elias Mountains approximately 70 km southwest of the Tatshenshini-Alsek River junction (Mudie et al. 2005). It lies within the Sub-arctic Highlands Ecodivision of the Northern Boreal Mountain Ecoprovinces (Demarchi 1996 as cited in Mudie et al. 2005).

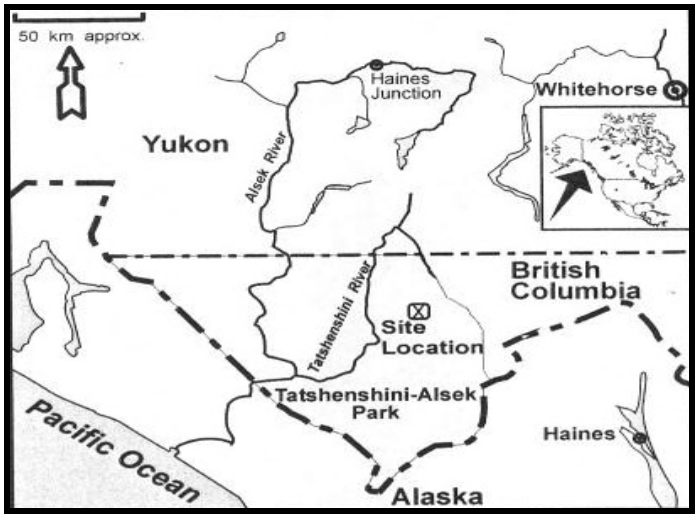

Figure 1.1. Kwäday Dän Ts'ìnchi site (map from Richards et al. 2007)

Artifacts recovered from the site included a wooden hand tool with an iron blade, a spruce root hat and an arctic ground squirrel robe. The hat and robe were initially radiocarbon dated 550 years BP, which is considered to be a time prior to the arrival of Europeans to the area (Beattie et al. 2000). A re-evaluation of the dates occurred when it was agreed that it was not a single-use site, and a clarification was also necessary on the appropriate marine reservoir correction. A radiocarbon analysis of a beaver skin bag sample that was in contact with the body and the inclusion of collagen samples from the individual resulted in a new estimated time-frame of AD 1670 to 1850 (Richards et al. 2007). This time period spans the transition from the end of pre-European times through to the advent of early European contact for the area.

The ancient individual was recovered on the traditional territory of the Champagne and Aishihik First Nations, and the elders named the site *Kwäday Dän Ts'ìnchi* meaning *long ago person found*. Pelvic morphology indicated that the individual was a male, and an assessment of epiphyseal fusion revealed that he was in his late teens or early twenties at the time of his death (Beattie et al. 2000). The frozen body was preserved but not intact, likely due to postmortem glacial movement. Additional remains, including the skull, were recovered in the years following the initial discovery, but they were not removed from the site (Royal British Columbia Museum 2008).

An agreement was reached between the Champagne and Aishihik First Nations (CAFN) and the British Columbia provincial government to allow scientific research on the artifacts and remains. It was important to the CAFN to learn more about this individual and determine his cultural ties. In the following decade, the research has helped to define the

individual, and it has also led to a renewed interest in maintaining the cultural knowledge from the past.

Southern Tutchone is the aboriginal language of the CAFN, and traditional stories in the coastal Tlingit and Southern Tutchone languages speak of individuals who have lost their lives travelling over the glaciers (Swanton 1908 as cited in Richards et al. 2007). Through stable isotope analysis, Richards et al. (2007) determined that Kwäday Dän Ts'ìnchi had a marine diet for most of his life, but they identified that the individual had recently switched to a diet of terrestrial food. Corr et al. (2009) expanded on this research and through an analysis of the individual's bone and skin amino acids, they also determined that the bone signal indicated a marine diet. The skin sample indicated a shift to a terrestrial diet in his last few months.

At the University of Saskatchewan, a research team was formed that consisted of Dr. E. Walker from the Archaeology and Anthropology Department, Dr. H. Deneer from the Department of Pathology and Laboratory Medicine, and myself. A proposal was developed to study the microbial DNA associated with the ancient tissues. This dissertation will focus on the research that stemmed from the initial proposed study to identify genetic evidence of normal bacterial flora or pathogenic bacteria.

The tissues that were obtained from the September, 2000 autopsy of the ancient individual included samples from the stomach, small intestine, cecum, descending colon, lung, mediastinal lymph node, the right lobe of the liver, myocardium, section of the right fourth rib, skin from a fold on the lower left arm, putative spleen tissue, as well as samples of the stomach contents and colon contents. After initial attempts of using polymerase chain reaction (PCR) with primers designed to amplify any ancient microbial DNA were

unsuccessful because of amplification of DNA in the negative PCR controls, I chose to test the tissues for evidence of specific bacteria, namely *M. tuberculosis* and *H. pylori* even though the pathological and histological examinations by the pathologist did not uncover any evidence of infectious disease.

Prior to the analysis of Kwäday Dän Ts'ìnchi tissues, molecular studies confirming the existence of the disease tuberculosis prior to the arrival of Europeans to the New World had been published (Salo et al. 1994, Braun et al. 1998), but there was no molecular or osteological evidence of precontact tuberculosis on the Northwest Coast. Researchers have estimated that in the preantibiotic era only five to seven percent of *M. tuberculosis* infections resulted in osteological changes (Aufderheide and Rodriguez-Martin 1998). Currently one in three individuals is infected with *M. tuberculosis*, and with the high rates still a concern especially for northern First Nation communities, I felt it was important to specifically test the ancient individual for molecular evidence of an *M. tuberculosis* infection.

The initial target was the IS*6100* insertion sequence known to exist in multiple copies, and it is specific for the *M. tuberculosis* complex. If amplification of this region was successful, I developed a plan to further characterize the ancient strain through amplifications in the mycobacterial genome including a region within *Rv3479* to confirm specifically that the infection was *M. tuberculosis* and not *M. bovis*. *M. bovis* is similar to *M. tuberculosis* and part of the *M. tuberculosis* complex, but an *M. bovis* infection can be transmitted in infected milk and meat whereas *M. tuberculosis* can only be transmitted through an inhalation of the bacteria from an aerosol created by the cough from an infected individual. Further analyses of the *katG* and *gyrB* genes were also planned because of the

association between the variability of *katG* and host populations, and the connection between *gyrB* and antibiotic resistance.

Modern studies on *Helicobacter pylori*, the causative agent of gastritis and gastric cancer, indicate that currently one in two individuals are infected world-wide, especially in developing countries. While most infections do not result in clinical symptoms, fifteen percent of infected individuals are impacted by the presence of the bacterium (Atherton 2006). Studies of aboriginal northern populations reveal high infection rates and antibiotic resistance (Demma et al. 2008, Goodman et al. 2008). I chose to test the stomach tissue of the ancient individual from the Kwäday Dän Ts'ìnchi site for evidence of an *H. pylori* infection by using PCR to amplify regions of the *vacA* and *cagA* genes that are associated with the bacterium's virulence. At the time of this study, there were no publications on the amplification of ancient *H. pylori* DNA. Phylogenetic studies on modern DNA indicate that a vast number of modern *H. pylori* strains exist (Yamaoka et al. 2008). When modern sequences are compared, evolutionary patterns can be seen related to the movement of ancient human populations (Falush et al. 2003). This is possible given the knowledge that *H. pylori* is normally transmitted within families, especially between mother and child (Weyermann et al. 2006).

The Kwäday Dän Ts'ìnchi site is located on the traditional territory of the Champagne and Aishihik First Nations, and osteological and mitochondrial evidence indicated that the ancient individual was of First Nations ancestry (Monsalve et al. 2002). Through the analysis of a putative *H. pylori* gastric infection, there was a possibility that the characterization of an ancient strain associated with an Aboriginal individual on the Northwest Coast could reveal evidence of the extent of the bacterium's virulence and lead

to answers of why modern northern Aboriginal populations have high infection rates. Also, since modern *H. pylori* genetic studies have been associated with the study of past migration, it was constructive to determine if an ancient North American *H. pylori* strain had any connection with modern Asian strains.

As a lead-in to the research, chapter two will be a review of the pertinent literature regarding the use of molecular technology, specifically PCR, in archaeological and physical anthropological studies. This section will include a review of microbial DNA studies of mummified individuals, in particular the Tyrolean Iceman. I will also provide a short review of the study of ancient pathogens, which will be followed by a brief overview of the study of past human migration into the New World. Chapter three covers the identification of *Helicobacter pylori* in the stomach tissue of the ancient individual from the Kwäday Dän Ts'ìnchi site and the information gathered from the characterization of the ancient strain. Chapter four encompasses the research involved with the identification of *Mycobacterium tuberculosis* in the ancient individual's lung tissue. This chapter is followed by a discussion of the importance of testing mummified remains for genetic evidence of both *M. tuberculosis* and *H. pylori* infections as well as a discussion of some of the technical lessons learned, including issues that I had regarding microbial DNA contamination. Finally, I conclude with what was specifically learned from the analysis of the microbial DNA from the ancient individual's tissues, and I will expand on how this information can be used to develop further research into *M. tuberculosis* and *H. pylori* infections and the connection with past human populations.

CHAPTER TWO: Literature Review

Higuchi et al. (1984) first investigated the survival of DNA in museum specimens of extinct animals. The researchers cloned mitochondrial DNA (mtDNA) from the dried muscle of an extinct quagga and proved that DNA survival was possible under certain conditions (Figure 2.1). This was followed by a publication on the cloning of human DNA from a 2400 year old Egyptian child mummy (Paabo 1985). Ancient DNA research that followed these landmark papers included the incorporation of a new method of enzymatically amplifying DNA referred to as polymerase chain reaction (PCR).

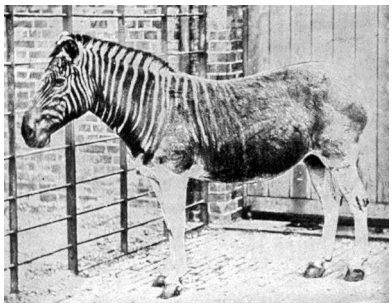

Figure 2.1. Quagga photograph taken in 1930 (www.life.com)

PCR revolutionized ancient DNA research. Since there are no DNA repair processes once an organism dies, the successful recovery of ancient DNA is determined by the amount of molecular degradation and the amount of co-extracted organic PCR inhibitors (O'Rourke et al. 2000). PCR is an ideal method for working with degraded DNA due to the sensitivity of the technique and the ability to start from a small number of template molecules from the DNA of interest. Unfortunately, this sensitivity can also result

in the potential amplification of modern DNA sequences. In this chapter I will review the main PCR concepts and its applicability to working with ancient degraded DNA, and I will give a brief overview of where the technology has been used to learn more about ancient pathogens, mummified individuals and also how ancient DNA can add to our understanding of past human migration.

2.1 Using molecular technology in archaeology and physical anthropology

Cooper and Poinar (2000) summarized the criteria that are critical for the authentication and substantiation of ancient DNA analyses. To summarize, the main suggestions are:

- the use of a physically isolated work area to prevent contamination from other amplified DNA
- the inclusion of negative extraction and PCR controls and the exclusion of positive controls
- appropriate molecular behaviour where there is an inverse relationship between the size of the PCR product and the amount of DNA that is amplifiable
- reproducibility of the data
- the use of cloning to identify signs of DNA damage
- replication of the results in an independent laboratory especially with unique or unexpected results
- biochemical preservation for indirect evidence of DNA preservation

- quantitation of the DNA target especially when the target template is low in concentration
- identification of DNA in associated remains to indicate likely DNA survival in the sample of interest.

The field of ancient DNA research is more than two decades old and yet continually changing due to improved and faster technology. We are in a genomic era with the capability of retrieving DNA sequence information at rates unheard of when the field of ancient DNA first began. Current methodologies that incorporate the process of high throughput sequencing (HTS) enable investigators to amass data that has the potential to answer many anthropological questions such as where and when our human ancestors first evolved, and how did infectious disease affect past human populations. The following review papers provide high level overviews of the field as well as the potentials and pitfalls of ancient DNA research: O'Rourke et al. (2000), Kaestle (2002), Paabo et al. (2004), and Willerslev and Cooper (2005).

2.1.1 Ancient DNA Extraction

Over the past two decades, various extraction techniques have been employed with varying levels of success. Often the more successful method is dependent on the sample's environment, and for example, if the sample was recovered from soil, the extraction method must include a means of removing polymerase inhibitors (Cipollaro et al. 2005). One popular method incorporates the use of phenol and chloroform to purify DNA through the

removal of proteins, but researchers discovered that less DNA was recovered (Castella et al. 2006).

A simple extraction method developed by Yang et al. (1998) involves the use of QIAquick™ silica spin columns from a Qiagen™ PCR Purification Kit (QIAGEN Inc., Mississauga, Ontario) for DNA binding. The advantage of this technique is that the extraction protocol results in optimal recovery of DNA without also extracting PCR inhibitors. Proteins, salts and nucleotides flow through the silica spin columns but DNA molecules larger than 100 bp and smaller than 10 kb are retained, and once eluted from the columns the DNA is in an optimal condition for use in PCR. The suggested extraction buffer for digestion of the samples prior to the use of the silica columns consists of 0.5M EDTA, 0.5% sodium dodecyl sulfate, which is a detergent to disrupt cell membranes, and 100 µg/ml proteinase K for protein digestion (Yang et al. 1998).

Rohland and Hofreiter (2007) compared the different methods used for DNA extraction and identified that a minimalist approach was more successful. They showed that the use of basic chemical components such as ethylenediaminetetraacetic acid (EDTA), a chelating agent that sequesters cations and suppresses the activity of nucleases that damage DNA, was beneficial. They also commented on the importance of proteinase K for the digestion of protein and removal of contaminants. They discovered that the addition of guanidinium, which is a chaotropic agent that denatures proteins, before the binding of DNA to silica was ideal for DNA purification. They also noted that bovine serum albumin (BSA) was very helpful in controlling inhibitors (Rohland and Hofreiter 2007).

2.1.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an enzyme based reaction that exponentially amplifies DNA. The components of a PCR reaction include:

- template DNA (a DNA molecule is a nucleotide that consists of one of four nitrogenous bases - adenine, guanine, cytosine and thymine, a five-carbon sugar - 2'-deoxyribose, and a phosphate group) (Figure 2.2)
- deoxyribonucleotide triphosphates (dNTPs)
- polymerase
- buffer for pH stability
- magnesium chloride ($MgCl_2$) which is required by the polymerase
- primers

Primers refer to oligonucleotides that are specially designed to bind to known stretches of DNA. A critical feature of a primer is the melting temperature (T_m), which is the temperature where half of the DNA molecules are single-stranded and half are double-stranded. When the melting temperature is incorrectly estimated, nonspecific products may be amplified (Korbie and Mattick 2008).

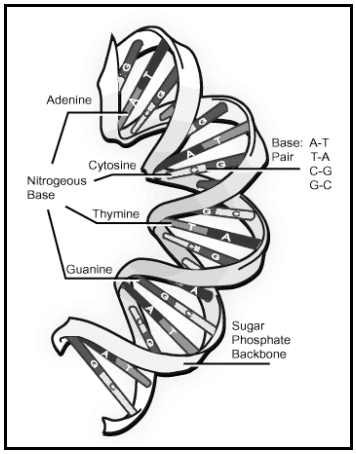

Figure 2.2. The DNA Double Helix (www.scq.ubc.ca)

The methodology involves three key processes; denaturation of the sample DNA, the annealing of oligonucleotides (primers) to the DNA, and extension of the newly synthesized DNA strand via the action of a polymerase that incorporates the correct free nucleotides. The process takes place through a temperature cycle such that the double-stranded DNA denatures at a high temperature, which is followed by the annealing of the primers at a lower temperature, and finally a return to a higher temperature for the extension of the newly synthesized DNA (Figure 2.3).

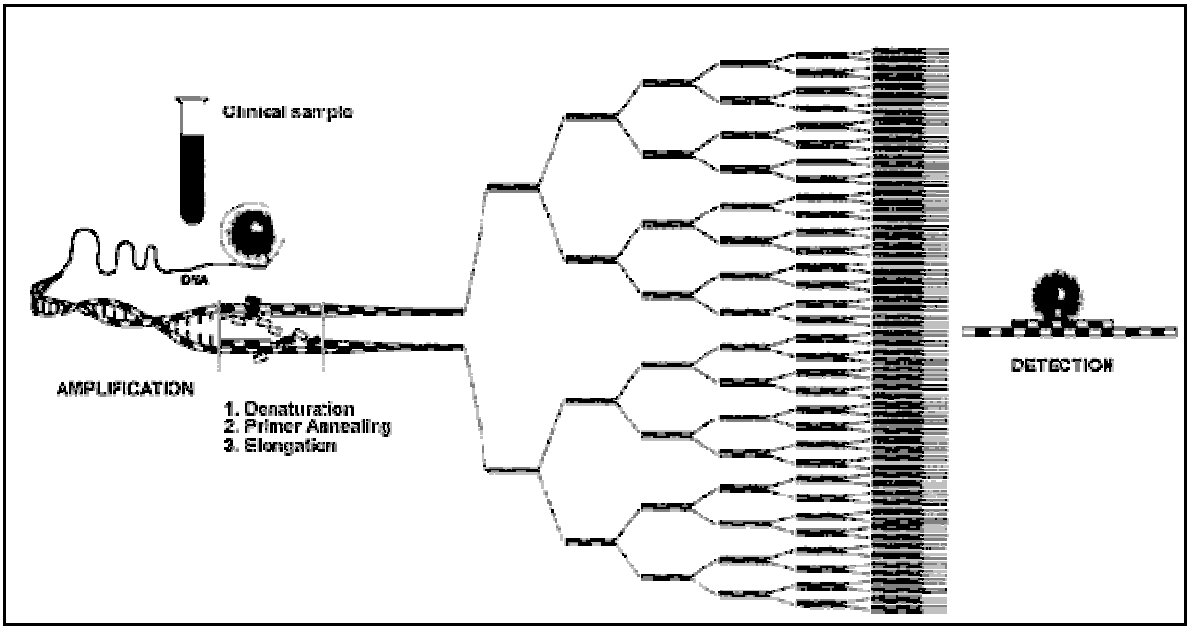

Figure 2.3. Schematic representation of the polymerase chain reaction (www.bioscience.org)

A few important rules are required for the development of a primer. The optimal primer length is approximately 18 to 22 bases, which is chosen for the ease of binding to the complementary DNA strand but it has enough specificity to allow the amplification of the DNA fragment in question. Successful primers should have a melting temperature in

the range of 52˚C to 58˚C, and the ideal guanine and cytosine content of the primer is between forty and sixty percent.

Rohland and Hofreiter (2007), after comparing the efficiency of 11 polymerases on DNA extractions from European cave bear bone and teeth samples, determined that the AmpliTaq Gold™ polymerase (Applied Biosystems) was the most suitable for amplification of ancient DNA. AmpliTaq Gold™ polymerase has been included in numerous studies that involved the amplification of ancient DNA mainly due to the high specificity and efficiency that is achieved as a result of the hot-start and time-release capabilities of this enzyme (Yang et al. 2003).

2.1.3 The difficulties of working with damaged DNA

Amplification of ancient DNA is most successful when the target sequences are less than 200 basepairs (bp). Postmortem DNA is fragmented due to varying levels of oxidation and hydrolysis. Taphonomic factors that affect an organism's DNA after death include temperature, water content and oxygen content (Lamers et al. 2009). The chance of DNA recovery is not based on age of the sample in question but on the environment affecting the sample with cool and dry conditions the most ideal for DNA preservation (Donoghue et al. 2004).

Miscoding DNA lesions result from different types of DNA damage. The most common miscoding lesion is the deamination of cytosine to uracil due to hydrolytic damage. This leads to a cytosine to thymine transition upon amplification (Paabo et al. 2004) (Figure 2.4). The enzyme uracil-N-glycosylase is normally used to prevent carryover contamination from uracil-containing DNA, but it may also be used to eliminate

the amplification of damaged sample DNA with deaminated cytosines. Unfortunately, because deamination of cytosine can be common, the use of uracil-N-glycosylase results in a reduction of the already short sample template DNA (Axelsson et al. 2008).

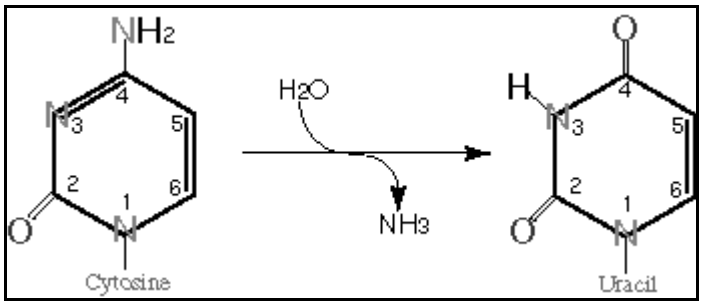

Figure 2.4. Cytosine deamination (http://asajj.roswellpark.org/huberman/DNA_Repair/damage_types.html)

DNA damage can result in single and double-strand breaks, structural modifications and protein crosslinks (Shapiro 2008). The known reduction in size of postmortem DNA is the result of many processes such as the hydrolytic cleavage of the phosphodiester bonds in the sugar-phosphate backbone (Paabo et al. 2004). Abasic sites can also be created from hydrolytic cleavage of the glycosidic bond between the sugar and base (Paabo et al. 2004). Other types of damage result in the inability of the polymerase to continue along the DNA strand during the formation of a complementary strand. Blocking lesions are often the result of peroxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl (OH) radicals (Paabo et al. 2004) (Figure 2.5).

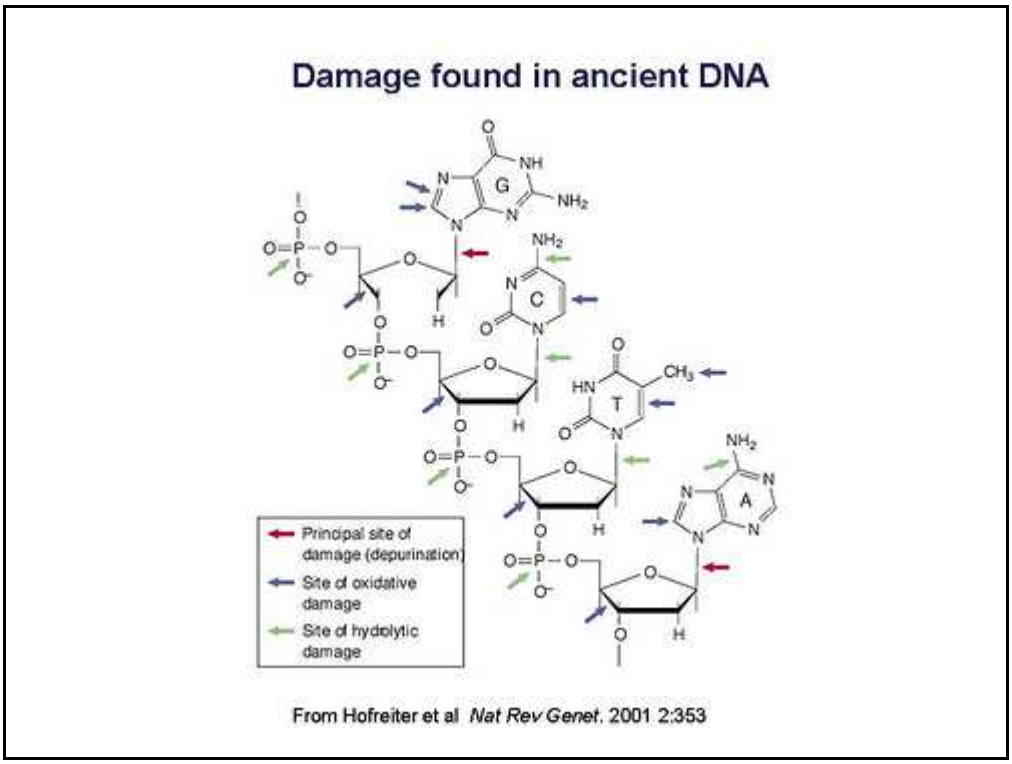

Figure 2.5. DNA damage sites (www.network.nature.com)

Damage can also occur as a consequence of chemical reactions between sugars, proteins and nucleic acids that result in Maillard products. Suggested solutions for this type of damage include the use of N-phenacylthiazolium bromide (PTB), which breaks apart the Maillard products and results in the exposure of the DNA to be amplified (Paabo et al. 2004).

2.2 Ancient pathogens – the search for evidence of past infectious disease

The study of ancient microbial DNA (paleomicrobiology) is still a relatively new field and first began with the identification of *Mycobacterium tuberculosis* DNA in osteological samples from Europe, Borneo and Turkey (Spigelman et al. 1993). With the involvement of molecular techniques, paleopathologists now have an additional resource

for the study of ancient disease. Through the analysis of the DNA that was associated with ancient pathogenic bacteria, it is now possible to confirm paleopathological diagnoses that were once based solely on the appearance of the skeletal material. The field also adds further information on the spatial and temporal relationships of past diseases and their impact on human populations. Researchers believe that this may help with understanding the evolution of modern diseases. The identification of sequence changes in ancient microbial DNA reveals evidence of genetic evolution in real time instead of relying on calculations based on the concept of a molecular clock (Donoghue et al. 2004).

The analysis of pathogenic microbial DNA is important to scientists who are defining the roles that the associated bacteria play in the development of specific human diseases. Certain infectious diseases will leave osteological evidence, but this percentage is small. Tuberculosis is one such disease that with time may affect skeletal bone, but even in the pre-antibiotic era, skeletal involvement was identified in only three to six percent of the cases (Donoghue and Spigelman 2006). One challenge of identifying the causative agent of a disease process involves deciding which tissue samples have the most likelihood of containing the microbial DNA, because the distribution of micro-organisms is not even between tissues. Occasionally dissemination of the bacteria occurs during the course of an infection, therefore before any attempts are made at amplifying ancient microbial DNA, a thorough understanding of the bacterium and the resulting disease process is required.

Since the emergence of paleomicrobiology, a variety of ancient bacterial studies have been published and reviewed (Zink et al. 2002, Drancourt and Raoult 2005, Roberts and Ingham 2008). Scientists have both successfully and unsuccessfully studied ancient DNA from many pathogenic micro-organisms. Some of the these investigations included

the study of DNA from *Mycobacterium leprae*, the causative agent of leprosy; *Treponema pallidum*, the bacterium associated with syphilis (Kolman et al. 1999, von Hunnius et al. 2007); *Yersinia pestis*, the causative agent of the plague (Drancourt et al. 1998, Gilbert et al. 2004, Wiechmann and Grupe 2005); *Trypanosoma cruzi*, a protozoan parasite responsible for Chagas' disease (Guhl et al. 1999) and *Corynebacterium*, the bacterium associated with diphtheria (Zink et al. 2001b). Viral studies have also revealed detailed information regarding ancient infections with the most notable work on the influenza virus that was involved with the 1918 flu pandemic (Reid et al. 2000, Taubenberger et al. 2001, Reid et al. 2004b, Reid et al. 2004a, Taubenberger et al. 2005).

The study of ancient tuberculosis remains the most common research topic in paleomicrobiology (Donoghue et al. 2004, Zink et al. 2007). Spigelman and Donoghue (2003) reviewed the important reasons why mycobacterial infections, especially those resulting from *M. tuberculosis*, are appropriate for ancient DNA studies:

- possible impact to bone as a result of the infection
- obligate pathogen with no environmental reservoir
- active infections result in tissue damage and caseation that protects the mycobacteria from postmortem spread of bacteria
- protective and durable mycolic acid cell wall
- the existence of many PCR-based identification methods and international databases
- the complete genome has been sequenced.

These reasons support the continued research into the definition of the spatial and temporal extent of the disease tuberculosis.

As a result of the increase in paleomicrobiology-related research, Drancourt et al. (2005) reviewed the publications to determine the quality of the data and results. Good evidence was based on the detection of one or more original identifying sequences or the detection of two unrelated identifying sequences or the detection of one identifying sequence and one unrelated biological molecule. They also analyzed how the evidence was obtained, and they identified that the best quality was the result of two independent research teams using agreed protocols. Some of these protocols included only using laboratories free of the bacterial target in question, using negative controls and omitting positive controls, and analyzing several molecular targets (Drancourt and Raoult 2005).

Cooper and Poinar (2000) and Paabo et al. (2004) have outlined acceptance criteria for maintaining confidence in results that stem from ancient DNA analyses. Roberts and Ingham (2008) reviewed 65 papers related to the identification of ancient pathogens from 29 journals that dated between 1993 and 2006. They discovered that most of the papers they reviewed did not detail how the acceptance criteria were followed or reasons for why only certain criteria were followed. They also noted that while the standard of research has improved over time, there are still inconsistencies that have resulted in continued concerns regarding the publication of paleomicrobiology papers with data that resulted from contamination.

The criteria that Roberts and Ingham (2008) based their analysis on included: sterile sampling during the initial excavation, a physically isolated and dedicated work area, and the development of a DNA history for personnel and past studies in the laboratory. Further recommendations included the use of protective clothing, removal of sample surface contamination, the assessment of mitochondrial DNA and nuclear DNA survivability, and

the use of negative extraction and amplification controls with the reporting of contamination. Evidence of appropriate molecular behaviour (PCR success is inversely related to product size) should be evident. Also, when compared with other relevant DNA sequences, the sequence under study should make phylogenetic sense. The results need to be reproducible from the same extraction sample or different samples, PCR sequences should be verified by cloning amplified products, and an independent laboratory should be involved to confirm data in situations where the results were unexpected. Lastly, the biochemical preservation of other sample components should be determined, the DNA target copy number quantified, and DNA survival in associated remains at the site should be analyzed (Roberts and Ingham 2008).

2.3 Mummified individuals

Mummified individuals have always held a fascination because they represent people who had lived in an earlier time and faced different challenges. The soft tissue preservation is sometimes great enough that the tissue has recognizable features, and this preservation results in the possibility of additional research beyond what is possible when skeletal remains are analyzed. Investigators have incorporated various methodologies into examinations of mummified tissues but recent biochemical and molecular developments have resulted in the opportunity to study past infectious diseases through an analysis of the microbial DNA associated with the tissues (Lynnerup 2007).

Zink et al. (2000) analyzed the remains of an ancient Egyptian infant mummy from the third intermediate period (1000 to 700 BC) and identified signs of anemia and vitamin C deficiency. The investigators obtained a metatarsal bone for DNA extraction and

amplified bacterial DNA using primers designed to amplify 16S ribosomal DNA. They uncovered evidence of bacteremia with the identification of DNA from pathogenic bacteria that included *Escherichia coli*. They suggested that due to the infant's anemia and vitamin C deficiency, a gastrointestinal infection may have disseminated and contributed to the infant's death (Zink et al. 2000).

When larger populations of mummified individuals are available, epidemiological studies can be undertaken through a combination of pathological, histological and molecular analyses. Recently, populations of Egyptian and Hungarian mummies have been studied with a focus on obtaining molecular evidence of infectious disease, especially tuberculosis (Zink et al. 2001a, Fletcher et al. 2003). Zink et al. (2001) analyzed tissue samples from ancient Egyptians dating between 3000 BC and 500 BC, and they confirmed through molecular analyses the presence of tuberculosis in this ancient population. Fletcher et al. (2003) used genotyping techniques to further characterize the *M. tuberculosis* strains associated with three individuals within the Hungarian mummy population. Through the analysis of multiple individuals in both studies, the impact of tuberculosis on those past populations is more thoroughly understood.

2.3.1 Tyrolean Ice Man

In 1991, hikers discovered human remains eroding out of a glacier on the Italian side of the Tyrolean Alps. The individual was not recovered using archaeological methods because the excavators assumed that the frozen individual was from the twentieth century (Fowler 2000). Through a complete investigation of the male individual, the scientists estimated that his age at death was 45 years old. Radiocarbon dating indicated that the site

was 5300 years BP. This time period is part of the Copper Age in Europe, which is associated with the first use of metal tools, and it is worth noting that a copper axe was recovered with the individual. In the years since his discovery, scientists continue to learn more about this ancient individual, popularly known as the *Iceman* or *Ötzi*.

DNA studies have been performed on both the individual's mitochondrial DNA (mtDNA) (Handt et al. 1994, Rollo et al. 2006, Ermini et al. 2008) and the microbial DNA associated with his tissues (Rollo et al. 2007, Cano et al. 2000, Rollo et al. 2000, Rollo et al. 1997). A histological analysis early in the investigation of the individual's tissues identified gram-negative bacteria in the digestive tract (Hess et al. 1998). This find was additional evidence of the existence of micro-organisms associated with the mummified tissue.

In 2000, Cano et al. published their analysis on the bacterial DNA in the colon and stomach of the Tyrolean Iceman. While the colon consisted of mainly fecal flora such as members of the genus *Clostridium*, the stomach contained evidence of microbial DNA from only one bacterium previously known as *Burkholderia* (gram-negative rods). The researchers suggested that this indicated that there was no postmortem relocation of the bacteria from the colon (Cano et al. 2000). They noted that the presence of *Burkholderia pickettii* may have been the result of postmortem water aspiration due to the amount of meltwater surrounding the body. Since this study was published, *B. pickettii* has been renamed *Ralstonia pickettii*, and while it is identified in wet environments, it is also a commensal in the oral cavity (Stelzmueller et al. 2006). Further investigations of *Ralstonia pickettii* have uncovered a connection to human infections and its role as a pathogen especially in individuals with underlying conditions (Ryan et al. 2006).

2.4 Genetic evidence of human migration into the New World

In this section, a connection between the analysis of ancient DNA and the study of past human migration will be discussed. This will include examples of genetic research that have incorporated the study of human DNA (mitochondrial and Y chromosomal data), parasite DNA and microbial DNA (bacteria and viruses) to determine the spatial and temporal movement of populations, with a specific focus on how this research is relevant to understanding how the New World was populated.

2.4.1 Mitochondrial DNA

Human mitochondrial DNA (mtDNA) is often the first target choice for the genetic analysis of ancient tissues because mtDNA is normally present in multiple copies (O'Rourke et al. 2000). A recent study of human remains recovered from On Your Knees Cave on Prince of Wales Island, Alaska with a radiocarbon date of 10,300 years BP revealed that the mtDNA associated with the individual was haplogroup D (Kemp et al. 2007). Individuals within a haplogroup share a common ancestor. Haplogroup D is one of the haplogroups associated with the founding population of the New World according to a study by Torroni et al. (1993).

New mitochondrial studies indicate the possibility of two groups entering the New World from Beringia around the same time, approximately 15 to 17 thousand years ago. Perego et al. (2009) analyzed the data from 69 entire mitochondrial genomes and discovered two rare Native American haplogroups. This led to the reasoning that the New World was likely populated along the Pacific Coast as well as the route through the ice-free corridor between the Laurentide and Cordilleran ice sheets (Perego et al. 2009).

2.4.2 Y chromosome DNA

Athapaskan languages are spoken throughout a large geographical area from the Canadian and Alaskan Subarctic to the American Southwest. MtDNA studies indicate a similar pattern that suggests migration from the Subarctic to the Southwest. Since mtDNA is maternally inherited, Malhi et al. (2008) studied paternally inherited Y chromosome DNA associated with individuals from 26 North American populations to identify the same pattern. They observed the presence of European admixture, which limited the amount of information that could be gathered from only analyzing the Y-chromosome data. Even with these limitations, the investigators concluded that there was a relationship between the Y-chromosome genetic data between Athapaskans in the Subarctic and the Southwest (Malhi et al. 2008).

2.4.3 Parasites

The analysis of genetic material associated with parasites is also relevant to research on how the New World was first populated. Parasites have had a long association with humans. Pinworms, hookworms and whipworms are known human parasites, but hookworms and whipworms have life cycles that require warm soil to proliferate, and researchers have stated that this would not have been possible if the only migration route to the New World was through Beringia (Araujo et al. 2008). Pinworm genetic sequences from New World archaeological sites suggested different origins for the strains, and this led researchers to conclude that a coastal migration also occurred (Araujo et al. 2008).

2.4.4 Bacterial and Viral DNA

Researchers have identified that certain human viruses such as the JC polyomavirus virus have had long-standing associations with humans. JC polyomavirus is a DNA virus from the family *Polyomaviridae* with an infection rate between 70 and 90%. After the initial infection, which is usually acquired in childhood, the virus persists through the individual's lifetime in the renal tissue. Different genotypes have been identified that correspond with distinct geographical populations (Pavesi 2005). Human population history can be reconstructed based on the genetic patterns of these viruses, similar to how mitochondrial and Y-chromosome genetic polymorphisms have identified geographical and temporal relationships between humans. The phylogenetic information from these viruses are representative of the human hosts that carry them (Ghose et al. 2002).

Researchers have recently discovered through phylogenetic analyses of modern bacterial isolates such as *Helicobacter pylori* and *Mycobacterium tuberculosis* that a connection can be made between bacterial DNA and the study of ancient human migration (Falush et al. 2003, Gagneux et al. 2006). A phylogenetic analysis involves the analysis of an organism's genetic sequences to determine evolutionary relatedness between individuals in a population. Phylogenetic trees are constructed to visually represent the branching pattern of the relatedness.

Modern *H. pylori* isolates from infections of individuals in the Amazon were analyzed in a recent study to determine if the bacterium had been present in the New World before the arrival of Europeans (Ghose et al. 2002). The investigators identified that East Asian genotypes were present in the Amazonian isolates thus confirming an ancient Asian connection with the indigenous population of the Amazon. Yamaoka et al. (2002) analyzed

1042 modern *H. pylori* isolates retrieved from aboriginal individuals. They chose strains that represented a large geographical area from Alaska, United States to Colombia in South America. Their findings suggested that *H. pylori* crossed the Bering Strait at different times (Yamaoka et al. 2002).

These studies are interesting because they indicate that we can learn about past human populations through a phylogenetic analysis of modern bacterial sequences, but even more exciting is the realization that a genetic analysis of ancient bacterial DNA can be used to identify past population movement. The use of molecular technology to identify evidence of microbial DNA in mummified individuals is continuing to evolve and will continue to be relevant to both paleopathology and archaeology.

CHAPTER THREE:

Evidence of an Ancient Gastric Pathogen: The Characterization of *Helicobacter pylori* DNA Associated with Frozen Human Remains Recovered from a Glacier in Northern British Columbia, Canada

ABSTRACT

Helicobacter pylori is a gram-negative bacterium that colonizes the stomach of half of the world's population. This pathogen causes gastritis in all infected individuals, but approximately fifteen percent of people infected with *H. pylori* also have clinical symptoms that may include peptic ulcers or gastric adenocarcinoma. Genotyping of *H. pylori* strains involves the analysis of the virulence-associated genes, namely *vacA* and *cagA*. Various *vacA* alleles have been identified in modern strains, and the *cagA* gene is normally either present, truncated or absent. Previous phylogenetic analyses have revealed a connection between modern *H. pylori* strains and the movement of ancient human populations. In this study, *H. pylori* DNA was amplified from the stomach tissue of an ancient individual recovered from a glacier in northern British Columbia on the traditional territory of the Champagne and Aishihik First Nations. The site was given the name Kwäday Dän Ts'ìnchi by the Aboriginal elders, and radiocarbon dates indicated a timeframe of approximately AD 1670 to 1850. This is the first ancient *H. pylori* strain to be characterized with *vacA* sequence data. The *H. pylori* strain has a unique hybrid *vacA* m2a/m1d middle (m) region.

This is significant because modern m1/m2 hybrid strains have been identified in Asian strains. Phylogenetic analysis indicated that the *vacA* m1d region of the ancient strain clusters with previously published novel Native American strains that are closely related to Asian strains. This indicates a past connection between the ancient individual from the Kwäday Dän Ts'ìnchi site and the ancestors who crossed the Bering Strait thousands of years ago. The ancient *H. pylori* strain was also indentified to contain a *vacA* s2 allele in the signal (s) region. This allele is more commonly identified in Western strains, suggesting that European strains were present in northwestern Canada during the ancient individual's time. Modern infection rates of Aboriginal individuals in the circumpolar regions are higher than average, and some treatments have been unsuccessful due to antibiotic resistance. Modern strains with *vacA* s2 alleles are often associated with limited or no impact to the individual's health. The identification of the *vacA* s2 region in the ancient strain suggests that the individual did not suffer from any symptoms due to the infection. The *cagA* status could not be confirmed due to the lack of amplification in that region. A portion of the *flaA* gene associated with the bacterium's flagella for motility was also amplified and sequenced. This ancient *flaA* sequence was identified as highly similar to the same region in Shi470, which is a modern strain that was isolated from an individual who resides in a remote Amazonian village in Peru. This corresponds with the *vacA* m region data that suggests a connection with ancestors who travelled from Asia.

3.1 INTRODUCTION

3.1.1 The role of *Helicobacter pylori* in human health

Helicobacter pylori is a helical, gram-negative, microaerophilic bacterium that inhabits the stomach of more than 50% of the world's population (1). The bacterium *H. pylori* is one of many bacterial microbiota that are able to colonize the human stomach (2). It survives the low pH environment through the generation of an enzyme urease which breaks down urea to ammonia and carbon dioxide (3). The bacterium also has flagella for motility that enable it to concentrate within the protective mucus layer of the stomach (3). It was first discovered over one hundred years ago when gastric bacteriologists Bottcher and Letulle identified bacterial colonies in ulcers and their mucosal margins (4), though it wasn't until 1983 when Marshall and Warren cultured the bacterium and made the connection with gastritis, an inflammation of the stomach (5) for which they received the Nobel Prize in Physiology or Medicine in 2005 (6).

The bacterium is now associated with peptic ulcers and gastric cancer, although most individuals remain asymptomatic (7). Approximately 15% of *H. pylori* infections result in peptic ulcers (1). Gastric cancer (gastric adenocarcinoma) is the second highest cause of cancer death because of its aggressive nature and the limitation of treatment options. Approximately 0.5% to 2% of individuals with an *H. pylori* infection will have gastric adenocarcinoma (1).

Scientists have discovered that *H. pylori* is mainly transmitted within families, especially from mother to child (8). This infection is normally acquired in childhood, and the bacterium's genetic fingerprint remains the same for decades (9). The highest risk

factor for acquiring the bacteria is through exposure to vomit, whereas it is quite rare for contraction of the infection through feces, saliva or the environment (3). *H. pylori* infections have decreased in regions with modern sanitation infrastructures whereas it is still a common infection worldwide (10).

3.1.2 Genetic characterization of *H. pylori*

H. pylori strains have been found to be genetically diverse. The complete genomes of five *H. pylori* strains have been sequenced: 26695, J99, HPAG1, G27 and Shi470 (11-15). Comparisons have been made between strains 26695 and J99 to determine the amount of variation. The overall organization of the two genomes is similar and the majority of the nucleotide differences are the result of synonymous substitutions (16).

It is widely accepted that the impact of a bacterial disease is dependent on three main factors: the host immune system, the virulence of the bacteria and the influence of the environment. The degree of *H. pylori* virulence is based on a form of vacuolating cytotoxin and the presence of a pathogenicity island (*cag*) that encodes a type IV secretion system (17). The vacuolating cytotoxin is the result of VacA expression, and the *vacA* gene is found in all strains. Different alleles of this gene exist and have been connected to the severity of the *H. pylori* infection. The *vacA* gene is composed of a variable middle region consisting of m1 and m2 alleles (18). The m1 allele has subtypes m1a, m1b, m1c and m1d (19-21) whereas the m2 allele has subtypes m2a and m2b (22). The m1 and m2 alleles have been found to differ in a 300 amino acid region by approximately 50% (23). Researchers have discovered that m1 is linked more often with disease because of the increased binding of the expressed VacA to cells (7).

The signal region of the *vacA* gene is also variable, and it consists of s1 and s2 alleles (18). Subtypes s1a, s1b and s1c have been identified in s1 strains. The s1 region is toxic, whereas in strains with the s2 allele, the mature toxin has an N-terminal extension that blocks vacuolation. All four middle and signal region combinations have been identified, but s2/m1 is rare (7). Strains with s1/m1 alleles produce high levels of toxin whereas little or no toxin is produced from s2/m2 alleles (24). The s1/m1 combination is more commonly associated with peptic ulcers and gastric carcinoma (25).

A second *H. pylori* virulence factor was determined to be CagA, which is associated with the presence of the *cag* pathogenicity island (PAI) (66). The presence of *cagA* is variable and is found in approximately 50% of the strains, and studies have shown that CagA positive strains are associated more often with severe disease (66). Researchers discovered that CagA is injected into the host cells via a type IV secretion system that is encoded by the *cag* PAI (67).

3.1.3 Phylogenetic analyses and past human migration

The genetic evolution of a bacterium represents the evolution of its human host if there is no horizontal transmission of the micro-organism (26). *Helicobacter pylori* fits this category and numerous studies have shown that the genetic differences in the *H. pylori* genome are equivalent to genetic differences in human populations. This has led to the analysis of population movement based on the genetic variation in a bacterium. There has been speculation that *H. pylori* has been associated with humans for thousands of years. Some of the evidence to support this includes the high genetic diversity of the bacterium and the presence of similar microorganisms in non-human primates as well as many other

animal species (27). Phylogenetic analyses based on strain sequence comparisons indicate that *H. pylori* likely made the move with their anatomically modern humans hosts out of East Africa around 58,000 years ago (28).

Researchers have identified a connection between the genotypes of modern *H. pylori* isolates and their geographical locations. Van Doorn and colleagues analyzed 611 *H. pylori* strains from patients in 24 countries and identified a geographical distribution of the *vacA* alleles. The *vacA* m1 and m2a alleles exist in equal frequencies except for Central and South America and Spain where the most prevalent allele is m1 (29).

Achtman and colleagues analyzed seven housekeeping genes and two virulence-associated genes from 20 *H. pylori* strains found in various locations around the world to gain a better understanding of the species diversity. High levels of synonymous variation were identified but clonal groupings were recognized (30). Analyses of the housekeeping and virulence-associated genes from an additional 370 strains revealed four modern populations: Africa1, Africa2, East Asia and Europe (31). Upon further analysis, Falush and colleagues defined subpopulations – HpEast Asia splits into the Amerind, East Asian and Maori subpopulations, and HpAfrica1 splits into West African and South African subpopulations.

Kersulyte and colleagues suggested that *H. pylori* became widespread recently within the evolution of humans based on various motifs identified at the right end of the *cag* pathogenicity island. The native Peruvian strains analyzed were more genetically similar to Spanish strains than strains from Asia. This led the researchers to suggest that the Peruvian strains were brought to the New World by the Spanish conquerors five hundred years ago (32).

Until recently, the only physical evidence that *H. pylori* was present in the New World prior to the arrival of Europeans consisted of the identification of *H. pylori* antigens in 3,000 year old fecal specimens (33). More recently, phylogenetic analyses with sequences from modern strains have been incorporated into studies to determine whether *H. pylori* was indeed present prior to European contact. Yamaoka and colleagues analyzed 1042 modern *H. pylori* isolates and identified novel *vacA* and/or *cagA* genes in eight Native Columbian and Alaskan strains. They identified that these sequences were closely related to sequences from East Asian *H. pylori* strains (21).

H. pylori infected gastric samples from individuals located in the Venezuelan Amazon were analyzed by scientists for additional evidence that *H. pylori* arrived in the New World when it was first populated by humans. The study revealed that East Asian genotypes were present in all of the loci examined and indicated that *H. pylori* has been associated with humans in the New World for thousands of years (34).

In 2008, Castillo-Rojas and colleagues identified *H. pylori* in a pre-Columbian male mummy approximately 50 to 60 years of age at death from the La Ventana burial cave in northern Mexico. They extracted DNA from the gastric remains and used a 16S rRNA probe to identify a 109 bp *H. pylori* fragment in two gastric samples. One sample was also positive for *ureB* (35).

In their landmark paper on tracing human migration with *H. pylori*, Falush and colleagues noted that the *H. pylori* Amerind subpopulation did not indicate any signs of genetic drift as was seen in the Maori subpopulation, where *H. pylori* likely underwent a genetic bottleneck that resulted in low genetic diversity. They commented that without

evidence of drift, *H. pylori* was likely introduced into the New World in large numbers of individuals or on multiple occasions (31).

Phylogenetic studies support the idea that *H. pylori* was in the New World prior to the arrival of Europeans, but some questions remain as to why some modern New World strains share a high similarity with European strains. Since some modern Peruvian *H. pylori* strains are highly similar to Asian strains, Devi and colleagues commented that while the evidence indicated *H. pylori* has had a long association with humans and crossed the Bering Strait with their human hosts, the Peruvian *H. pylori cag* pathogenicity island (PAI) was likely acquired by a European source because of the similarity with the PAIs in the European strains (36). They suggested that the *cag* PAI may have helped with colonization which explains why Peruvian strains have an eastern-like core but a western-type *cag* PAI. The PAI may have been acquired during a mixed infection but the question remains whether the entire PAI was exchanged (36). Studies have shown that most of the Amerind subpopulation isolates did not have a *cag* PAI and only a few isolates had a partial PAI (37).

3.1.4 *Helicobacter pylori* and ancient human remains

This study reports the identification and characterization of *H. pylori* DNA associated with the stomach tissue of the *Kwäday Dän Ts'ìnchi* ancient individual. In August 1999, three hunters discovered ancient frozen human remains in a glacier in northern British Columbia, Canada on the traditional territory of the Champagne and Aishihik First Nations. The site was given the name Kwäday Dän Ts'ìnchi by the Aboriginal elders, which means *long ago person found*. An agreement was made between

the provincial government and the Champagne and Aishihik First Nations to allow scientific analyses of the individual and the artifacts that were recovered from the site. Radiocarbon dates first suggested that the individual was approximately 550 years old (circa AD 1450), which was a time before the arrival of Europeans (38). Additional radiocarbon analyses were performed recently to clarify some original ambiguous results, and the researchers discovered that the individual dated to between AD 1670 and 1850 (39). Since European contact first occurred in 1741 between the Russians and the Tlingit on the Northwest Coast (68), this individual lived either at the end of precontact times or early in the European period.

Naturally mummified individuals are more suitable for bacterial DNA studies because they have not been impacted by any processes such as embalming that would alter the tissue environment (40). The confirmation of the presence of bacterial pathogens associated with ancient individuals is an important part of determining the temporal extent of infections affecting humans. *Helicobacter pylori* strains differ in virulence, and I chose to analyze genetic fragments of the strain's genome, including the virulence-associated genes *vacA* and *cagA* to determine the impact of the infection on the ancient Aboriginal individual. Also, because of the link between different *H. pylori* strains and past human migration, I attempted to determine if the strain associated with the individual had any similarities with Asian strains. This would add further evidence for the early presence of the infection in the New World.

3.2 METHODS

3.2.1 Sample Collection

A stomach sample from the Kwäday Dän Ts'ìnchi ancient individual was obtained in September, 2000 at the Royal British Columbia Museum in Victoria, Canada during the retrieval of tissue samples for multiple research teams. Standard protocols were followed to prevent contamination of the samples. All members of the autopsy team were dressed in appropriate protective clothing, and sterile surgical tools were used during sample collection. The stomach samples were frozen and the following day packed on ice in an insulated container for travel to the University of Saskatchewan, in Saskatoon, Saskatchewan, where they were stored in a -70°C freezer upon arrival.

3.2.2 DNA Extraction

Tissue extractions were set up in a biological safety cabinet that was surface cleaned with 10% (v/v) Clorox bleach. A sterile scalpel was used to mince 0.24 grams of stomach tissue into small fragments, and the DNA was extracted using the tissue protocol with the QIAamp® DNA Mini Kit (QIAGEN Inc., Mississauga, Ontario).

3.2.3 PCR Amplification

All PCR reactions were set-up in a separate location from the post-PCR laboratory. The components of the 50µl reaction consisted of: 5 µl DNA extract, 2 Units AmpliTaq® Gold DNA Polymerase (Applied Biosystems Canada, Streetsville, Ontario), GeneAmp® PCR Gold Buffer (15mM Tris-HCl, pH 8.0 and 50mM KCl), 2.5 mM $MgCl_2$, and 200µM

each dNTP from GeneAmp® dNTP Mix (Applied Biosystems Canada, Streetsville, Ontario). Previously published and newly designed PCR primers (0.2µM Sigma Genosys Canada, ON) were used for the amplification of the *Helicobacter pylori vacA* variable regions and portions of *flaA* (Table 3.1). Amplification within *cagA* and around the putative empty site was also attempted. PCR reactions were performed in an MJ Mini Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) as follows: 12 minutes initially at 95˚C, followed by 40 cycles of denaturation at 95˚C for 1 min, differing annealing temperatures for 1 min and extension at 72˚C for 1 min. After the 40 cycles were completed, the temperature remained at 72˚C for 8 minutes.

3.2.4 DNA Electrophoresis

Five microlitres of each amplification product were added to individual wells in 2% (w/v) agarose minigels that were immersed in a Tris/acetate/EDTA (1xTAE) solution. After the application of 88 volts for one hour, gels were removed and placed in a container containing a 0.5 µg/ml ethidium bromide solution for 10 minutes. The gels were destained in water for 10 minutes before visualization on a UV lightbox (254 nm).

TABLE 3.1: PCR primers for the amplification of regions within *vacA*, *cagA* and *flaA*

Region	Primers		Product size	Annealing Temp. Number of Cycles	Source
***vacA* s**	VA1F	ATGGAAATACAACAAACACAC	s1 176 bp	50˚C	18
	VA1XR	CCTGARACCGTTCCTACAGC	s2 203 bp	40 cycles	45
***vacA* m**	MF1	GTGGATGCYCATACRGCTWA[a]	m1 107 bp	50˚C	45
	MR1	RTGAGCTTGTTGATATTGAC[a]	m2 182 bp	40 cycles	45
	y98vacAmF	CCTTGGAATTATTTTGACGC	m1 479 bp	58˚C	46
	y98vacAmR	ATCCATGCGGTTATTGTTGT	m2 488 bp	45 cycles	46
	vacAmgapF	ATGCCAGCAAGAGCGATAAT	344 bp	52˚C	This study
	vacAmgapR	GCATTGTGGCCTAGGGTTAG		45 cycles	This study
cagA	cagAF	TTGACCAACAACCACAAACCGAAG	183 bp	50˚C	45
	cagAR	CTTCCCTTAATTGCGAGATTCC		40 cycles	45
	Ako982F	ACATTTTGGCTAAATAAACGCTG	360 bp	57˚C	59
	Ako9825R	TCATGCGAGCGGCGATGTG		45 cycles	59
	cagAnegF	GAGAGGGTGGTGCGATAAAA	236 bp	52˚C	This study
	cagAnegR	GGGCTATTTTATGGGGCATT		45 cycles	This study
flaA	flaA-1-F	AAAATCGGTCAGGTTCGTATCG	94 bp	62˚C	47
	flaA-2-R	CATCATTCACACCATCCACTTGTT		45 cycles	47
	flaA-3-F	AACAAGTGGATGGTGTGAATGATG	256 bp	62˚C	47
	flaA-4-R	GCGACTAACCTTCCGTCTGAGT		45 cycles	47

[a] R is A or G, W is A or T, and Y is C or T

3.2.5 DNA Sequencing and Phylogenetic Analysis

PCR products were sequenced directly in both the forward and reverse direction on an ABI 3730*xl* DNA sequence analyzer at the National Research Council-Plant Biotechnology Institute (Saskatoon, Saskatchewan). The DNA sequences were compared with reference sequences in the National Institute of Health (NIH) Genbank database, and phylogenetic analyses were used for the determination of evolutionary relationships. Dr. M. Haakensen of the Department of Food and Bioproduct Sciences at the University of Saskatchewan assisted with the phylogenetic analysis. The sequences were aligned with the ClustalX software program (41) and visualized and manually edited with the GeneDoc software program (42). All phylogenetic trees were produced with the Neighbour-Joining method and visualized using MEGA4 (Molecular Evolutionary Genetics Analysis software version 4.0) (43). Tree topology was evaluated using Minimum Evolution, Maximum Parsimony, and Unweighted Pair Group Method of Arithmetic Means algorithms. A bootstrap test (44) of 1000 replicates was performed.

3.3 RESULTS

3.3.1 *H. pylori vacA*

3.3.1.1 Proximal *vacA* m region

180 base pairs were amplified in the proximal *vacA* m region using previously published primers MF1 and MR1 (Table 3.1) on the stomach tissue extract (Figure 3.1). This region is subtype m2a and contains the sequence inserts that differentiate the m1 alleles from m2 (Figure 3.2). These primers were also used on additional Kwäday Dän

Ts'ìnchi tissue extracts, including the small intestine, descending colon and a skin sample, but no amplification occurred.

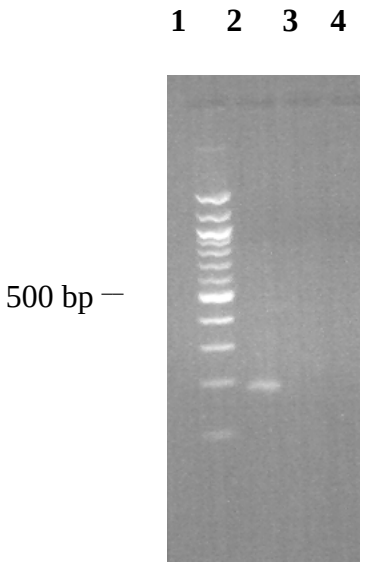

Figure 3.1. Amplified *vacA* m PCR product on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, Stomach extract amplified (180 bp) with previously published *vacA* m primers MF1/MR1 (45); **Lane 3**, negative extraction; **Lane 4**, negative PCR

GTGGATGCCCATACGGCTTATTTTAATGG**CAATATTTATCTGGGAAAATCCAC**
GAATTTAAAAGTGAATGGCCATAGCGCTCATTTTAAAAATATTGATGCCAGC
AAGAGCGAT**AATGGTCTAAACACT**ACCACTTTGGATTTGAGCGGCGTTACAG
ACAAGGTCAATATCAACAAGCTC

Figure 3.2. Amplified *vacA* m sequence with boxes around the inserts that define this allele as m2.

A phylogenetic analysis revealed a close relationship between a sequence of the Kwäday Dän Ts'ìnchi *H. pylori vacA* m region and *vacA* m sequences from strains isolated

in Okinawa, Japan (Figure 3.3) (24). The Japanese strains were classified into a Western cluster, and the authors suggested that this is an indication of contact with the West.

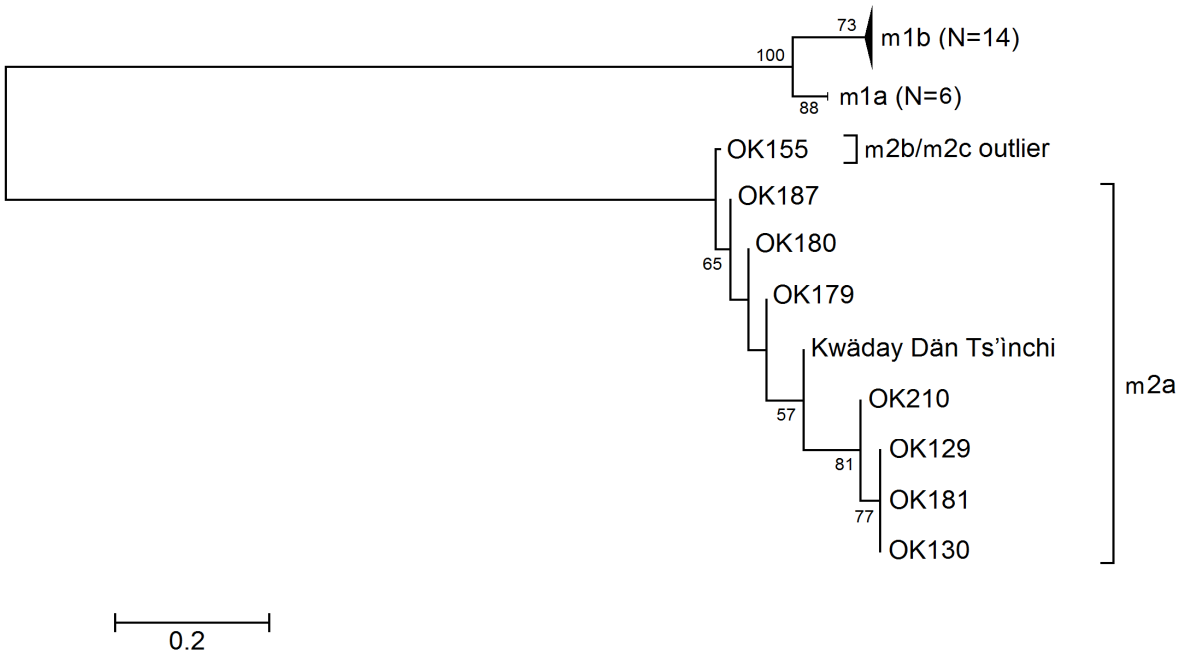

Figure 3.3. Phylogenetic tree of the m region of the *vacA* m gene (180 bp) that indicates the *H. pylori* DNA associated with the stomach tissue of the Kwäday Dän Ts'ìnchi ancient individual is type *vacA* m2a. All sequences in the tree are as described by Yamazaki et al. (24).

3.3.1.2 Distal *vacA* m region

483 base pairs were amplified in the distal *vacA* m region using previously published primers y98vacAmF and y98vacAmR (Table 3.1) (Figure 3.4).

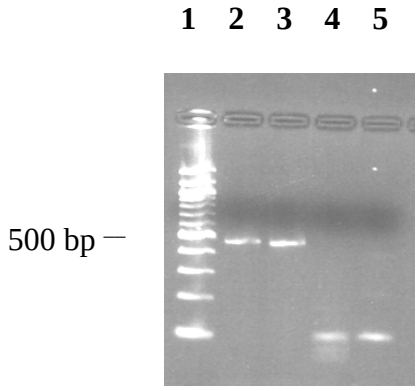

Figure 3.4. Amplified *vacA* m PCR products on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, Stomach extract amplified with previously published *vacA* m primers (46); **Lane 3**, Second stomach extract amplified with previously published *vacA* m primers (46); **Lane 4**, negative extraction; **Lane 5**, negative PCR

When the Kwäday Dän Ts'ìnchi *vacA* m distal sequence was compared with the *vacA* m region of modern strains, it clustered with m1d strains isolated from North and South American Aboriginal individuals in a study by Yamaoka and colleagues (21) on the presence of *H. pylori* in the New World prior to Columbus (Figure 3.5). No amplification resulted with newly designed primers for this study when the attempt was made to sequence the region between the two Kwäday Dän Ts'ìnchi *H. pylori vacA* m sequences (Table 3.1).

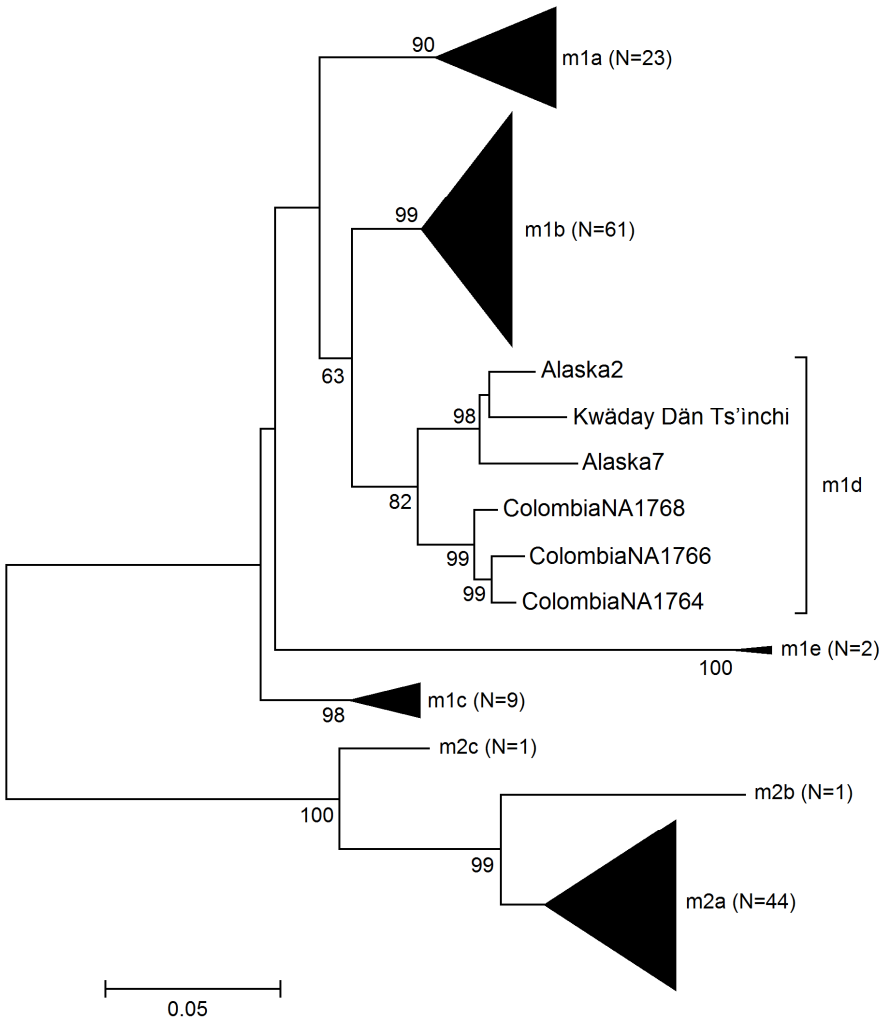

Figure 3.5. Phylogenetic tree of the *vacA* m region (483 bp) that indicates the *H. pylori* DNA associated with the Kwäday Dän Ts'ìnchi stomach tissue is type m1d. All sequences in the tree are as described by Yamazaki et al. (24) and Yamaoka et al. (21).

3.3.1.3 *H. pylori vacA* s region

203 bp in the *vacA* s region were amplified using previously published primers VA1F and VA1XR (Figure 3.6) (45). This sequence indicated that the Kwäday Dän

Ts'ìnchi *H. pylori* strain contained the *vacA* s2 allele with a type A signal region insert (Figure 3.7) (48).

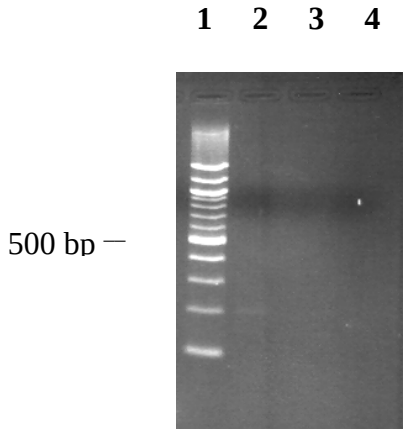

Figure 3.6. Amplified *vacA* s PCR product on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, Stomach extract amplified with previously published *vacA* s primers VA1F and VA1XR (203 bp) (45); **Lane 3**, negative extraction; **Lane 4**, negative PCR

ATGGAAATACAACAAACACACCGCAAGATCAATCGCCCTATTATCTCTCTCGCT
TTAGTGGGGGCATTAATTGGCACCGAACTGGGGGCTAATACGCCA**AATGATC**
CCATACACAGCGAGAGTCGCGCCTTTTTCACAACCGTGCTTATTCCAGCCATT
GTTGGAGGTCTCGCTACAGGAACCGCTGTAGGAACGGTCTCAGG

Figure 3.7. The *vacA* s region DNA sequence in the Kwäday Dän Ts'ìnchi *H. pylori* strain encodes a type A SR insert (NDPIHSESR) according to the 2007 study by Owen and Xerry (48).

A phylogenetic analysis indicated a close genetic relationship between the *vacA* s region of the Kwäday Dän Ts'ìnchi *H. pylori* strain and the *vacA* s regions from modern s2 strains isolated from North and South American Aboriginal individuals (Figure 3.8) (21). It is noteworthy to mention that the Kwäday Dän Ts'ìnchi site was discovered less than 50

km from the Alaska, USA border, which is interesting considering the phylogenetic analysis revealed that two modern Alaskan strains share a high percentage identity in the *vacA* s region with the strain associated with the Kwäday Dän Ts'ìnchi individual.

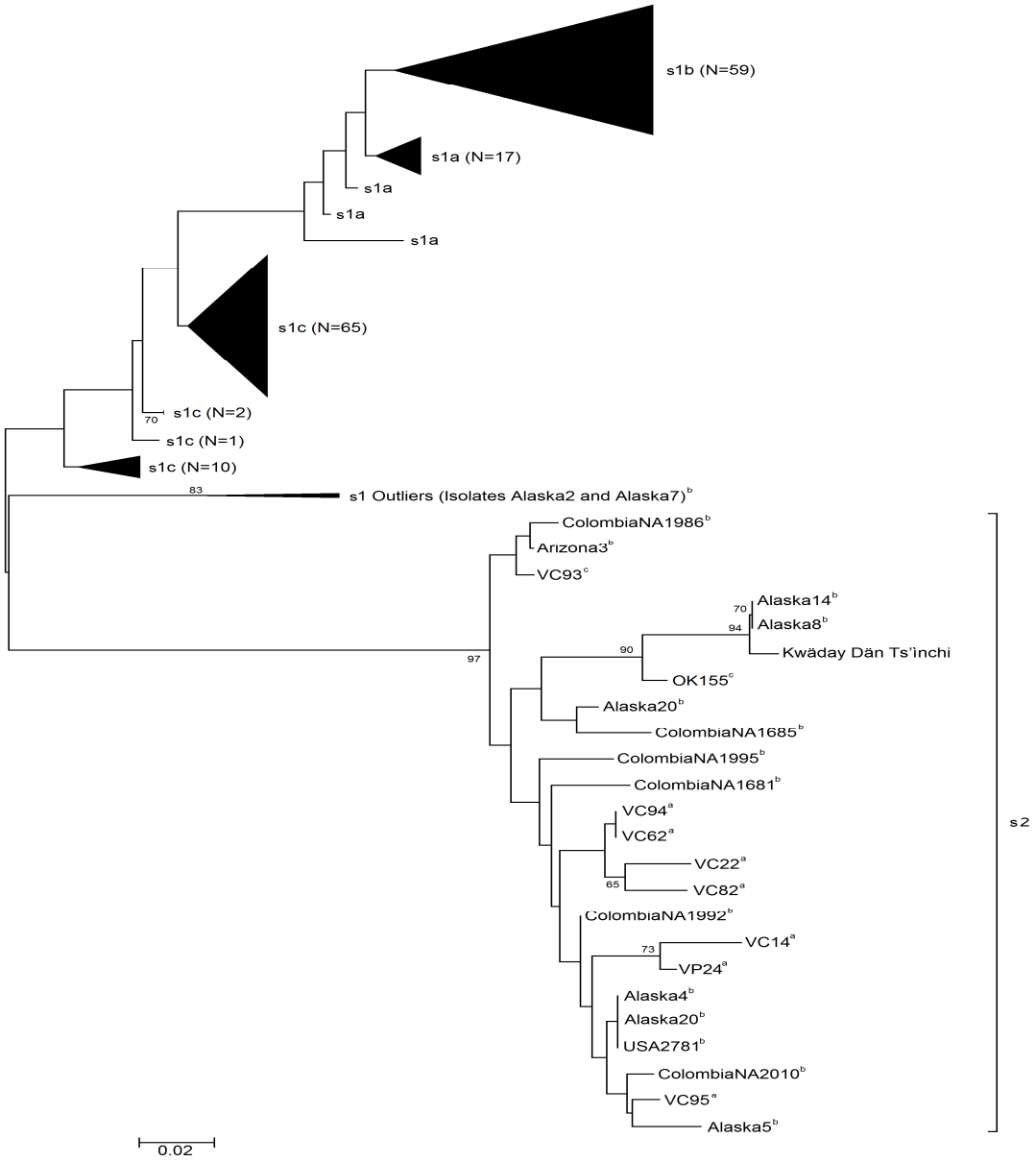

Figure 3.8. Phylogenetic tree of the s region of the *vacA* gene (203 bp) of *Helicobacter pylori* showing the relationship of *H. pylori* DNA associated with Kwäday Dän Ts'ìnchi stomach tissue with other documented strains of *H. pylori* ([a] 34, [b] 21, and [c]24)

Through the amplification and sequencing of a total of 866 bp from three locations in the *vacA* gene, the Kwäday Dän Ts'ìnchi *H. pylori* strain was characterized as *vacA* s2-m2a/m1d (Figure 3.9). These three locations included the *vacA* signal (s) region, a proximal portion of the *vacA* middle (m) region, and a distal portion of the *vacA* middle (m) region.

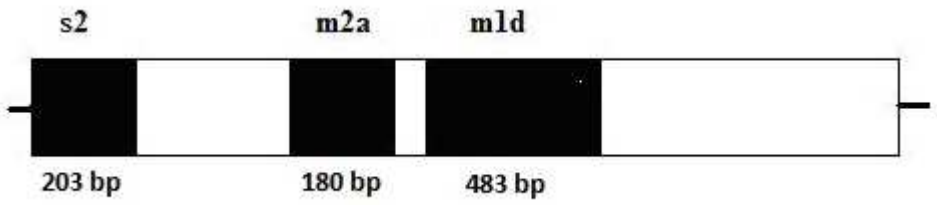

Figure 3.9. Kwäday Dän Ts'ìnchi *H. pylori vacA* gene with the regions sequenced in this study indicated in black

3.3.2 *H. pylori cagA*

Because both internal and flanking primers did not result in any DNA amplification, the ancient *H. pylori* strain's *cagA* status could not be confirmed.

3.3.3 *H. pylori flaA*

Using previously published primers flaA-3-F and flaA-4-R (47), 256 bp of the *flaA* gene was amplified from the Kwäday Dän Ts'ìnchi *H. pylori* strain (Figure 3.10) that corresponds to nucleotides 753574 to 753826 of strain Shi470. A phylogenetic analysis indicated that this sequence shares the highest identity (98%) with a partial *flaA* sequence from the Shi470 *H. pylori* strain (Figure 3.11). Two nucleotide positions were found to contain more than one base. If this is an indication of multiple alleles, the substitutions were synonymous (Figure 3.12).

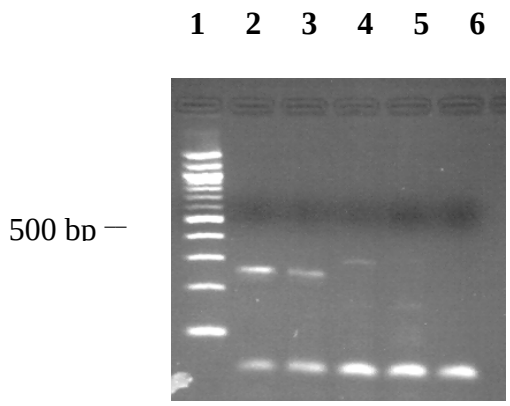

Figure 3.10. Amplified *flaA* PCR products on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, Stomach extract amplified with previously published *flaA* primers flaA-3-F and flaA-4-R (47); **Lane 3**, Second stomach sample amplified with previously published *flaA* primers (47); **Lane 4**, negative extraction; **Lane 5**, second negative extraction; **Lane 6**, negative PCR

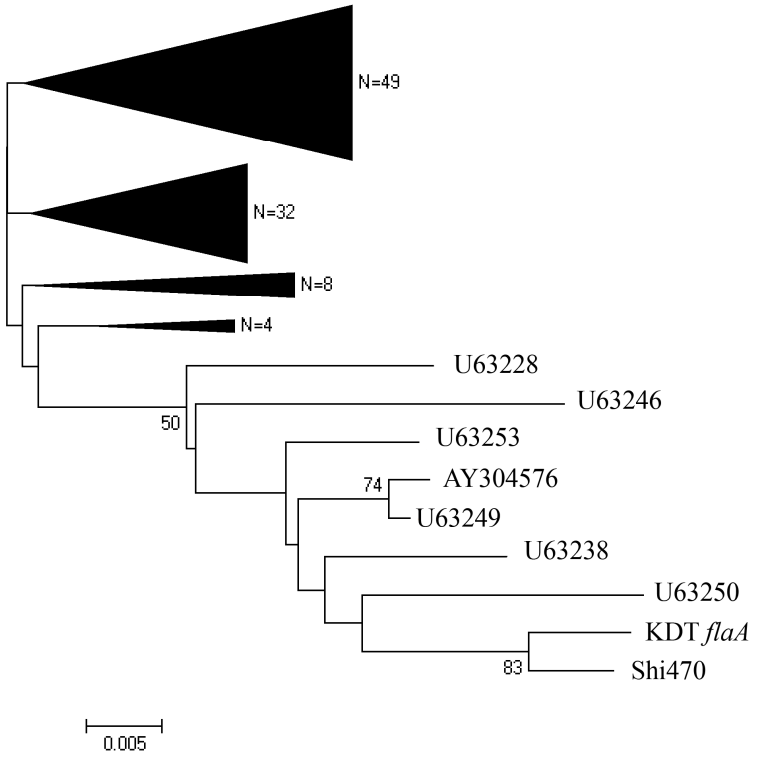

Figure 3.11. Phylogenetic tree of the Kwäday Dän Ts'ìnchi *flaA* gene sequence (256 bp) showing the relationship to other documented strains of *H. pylori*.

AACAAGTGGATGGTGTGAATGATGTAACTTTAGAGAGCGTGAAAATCTCTAGT
TCAGCAGGCACAGGGATTGGCGTGTTAGCGGAAGTGATTAACAAAAACTCTAA
CCAAACAGGGGTTAAAGCTTATGCGAATGTTATCACCACGAGCGATGTGGCGG
TCCA**A/G**TCAGGAAGTTTGAGTAATTTAACCTTAAA**C/T**GGGATCCATTTGGGC
AATATCGCAGACATTAAGAAAAACGACTCAGACGGAAGGTTAGT

Figure 3.12. Sequencing indicated that the amplified *flaA* fragment had two nucleotide positions with more than one base present.

3.4 DISCUSSION

3.4.1 Identification of an ancient gastric pathogen and relevance of the *vacA* characterization

Helicobacter pylori DNA was identified in the stomach tissue of the Kwäday Dän Ts'ìnchi ancient individual, and the strain was characterized as *vacA* s2-m2a/m1d. It is significant to find evidence of this ancient pathogen because other than a recent publication by Castillo-Rojas and colleagues (35), only modern *H. pylori* strains have been studied. While phylogenetic analyses suggested that this bacterial pathogen has been present throughout human history, the antiquity of this bacterium is further recognized in the analysis of actual ancient *H. pylori* strains.

3.4.1.1 *H. pylori vacA* proximal m region

The proximal m region of the ancient *H. pylori vacA* gene was characterized as m2a based on comparisons with modern *H. pylori* sequences in Genbank. Yamazaki and colleagues analyzed 220 *H. pylori* strains from Fukui and Okinawa, Japan to identify a

relationship between *vacA*, the *cag* PAI and the clinical outcome (24). None of the 115 strains from Fukui had a *vacA* m2 genotype, but 15 of the 105 strains from Okinawa were identified with the m2 allele. The researchers classified the m2 strains into a Western cluster and suggested that the appearance of the m2 allele in Okinawa was due to greater contact with the West. The Kwäday Dän Ts'ìnchi *H. pylori* strain is similar to these 'Western' Japanese strains in the *vacA* m proximal region, and this suggests that New World Aboriginal individuals were exposed to European *H. pylori* strains prior to AD 1850.

In a phylogenetic analysis of *vacA* genes from Chinese and Western isolates Ji and colleagues identified that the *vacA* m-region in m2 alleles was quite different from the rest of the gene indicating that this region was acquired by horizontal transfer (23). They also suggested that the *vacA* m2 m-region spread after the separation of Chinese and Western strains. They noted a contrast between the differences in the 5' m-region and the lack of divergence of the 3' m-region in m2 alleles suggesting an independent evolution of the two regions (23).

H. pylori vacA alleles are identified on the basis of short nucleotide sequence inserts within the signal and middle regions. Owen and Xerry (48) analyzed *vacA* sequences from 32 countries to determine a possible connection with the geographical location on insert diversity. They identified that the 75 bp middle region insert of 255 *vacA* m2 sequences was represented by 23 peptide variants. One predominant sequence was observed which defined 62% of the inserts. They also determined that middle region inserts were common in Western strains (European / North American) whereas East Asian sequences had a much lower insert frequency (Table 3.2).

The insert within the Kwäday Dän Ts'ìnchi *H. pylori vacA* m2 middle region is LKVNG HSAHF KNIDA SKSDN GLNTT, which was not identified in the Owen and Xerry study (48). The most similar m2 insert to the Kwäday Dän Ts'ìnchi strain insert was LKVNG HSAHF KNIDA SKSDN GLNTS found in two percent of the strains that were located in China, Taiwan, Peru and the Netherlands. The only amino acid difference between these inserts is a threonine (ACC) in the final position of the Kwäday Dän Ts'ìnchi insert whereas the most similar insert in the Owen and Xerry study (48) contains a serine (possible TCC codon). This difference may be the result of one base change (A to T). This could be an artifact of analyzing degraded DNA, but this is not likely because threonine has been identified in this final position of other *vacA* m inserts. This study has resulted in the identification of a novel *vacA* m insert associated with an *H. pylori* strain.

Table 3.2. *vacA* m region amino acid comparison between inserts from the Owen and Xerry study (48) and the insert from the Kwäday Dän Ts'ìnchi *H. pylori vacA* m region

% of strains	*vacA* m region amino acid insert	Location
62	LRVNG HSAHF KNIDA SKSDN GLNTS	Widespread
6	LKVNG HTANF KDIDA SKGRN GIDTT	China/Peru
2	LKVNG HSAHF KNIDA SKSDN GLNTS	China/Netherlands/Peru/Taiwan
	LKVNG HSAHF KNIDA SKSDN GLNTT	AD 1670-1850 northern BC glacier

3.4.1.2 *H. pylori vacA* distal m region

A total of 483 base pairs from the distal portion of the Kwäday Dän Ts'ìnchi *H. pylori vacA* m region was amplified using previously published primers (46). A

phylogenetic analysis of this sequence revealed that it was very similar to sequences from a study of modern Aboriginal North and South American *H. pylori* strains (21). They identified four (22%) Native Colombian strains with *vacA* m and *cagA* novel structures. Novel *vacA* m structures were also identified in four Native Alaskan strains (20%). They performed phylogenetic analyses on sequences of the *vacA* m region and the *cagA* gene of numerous *H. pylori* strains. Five Native American strains (Colombia-NA1764, -NA1766, -NA1768, Alaska-2 and -7) formed a cluster that was related to the East Asian *vacA* subtype m1b. They denoted this Native American subtype as m1d (21).

The *cagA* regions of the modern Native American isolates were also similar but not identical to the *cagA* region of East Asian isolates. One Alaskan strain (Alaska-2) was found to be *cagA* negative. Yamaoka and colleagues commented that these results indicated Native American *H. pylori* strains did not originate with modern East Asian people, but they likely had an ancient connection, which supports the theory that *H. pylori* was associated with the first humans in the New World (21). It is also interesting to note the connection between the strains from Alaska and the strains from Colombia. Their similarity suggests that they share a common Asian ancestor. The researchers noted that many of the Native American *H. pylori* strains have genotypes similar to those from non-Asian countries. They commented that this may indicate colonization differences between Old World and New World strains (21). The discovery of a similar *vacA* m region in the Kwäday Dän Ts'ìnchi *H. pylori* strain is significant because a connection has been identified between the ancient strain and local modern Alaskan strains as well as modern Asian strains.

3.4.1.3 *H. pylori* hybrid *vacA* m regions

While developing a new PCR-based typing system for untypeable *H. pylori* strains, Atherton and colleagues amplified the *vacA* m region of 77 strains from Asia, North and South America (49). An m1/m2 hybrid was identified. Strain Ch2 from China was found to have an m1-like sequence at the forward primer annealing site and m2-like sequence on the 3' end. The scientists examined the alignments of the Ch2 sequence with the sequences from both *vacA* m1 and m2 strains, and they identified a region containing the recombinational breakpoint (50). The Ch2 proximal *vacA* m region was type m1 whereas the distal section was type m2, which was likely the result of recombination (50). The breakpoint for Ch2 was identified at the coordinates of 1971 bp to 1985 bp in strain Tx30a.

In this study of the Kwäday Dän Ts'ìnchi *H. pylori* strain, two components of the *vacA* middle region were amplified. The proximal m region typed as m2a whereas the distal m region typed as m1d. The first 27 bases of the distal middle region amplified using the Yamaoka et al. study primers (46) is a region that is conserved between both m1 and m2 strains. At the breakpoint determined in the Atherton et al. study (50), the Kwäday Dän Ts'ìnchi *H. pylori vacA* sequence indicates an m1 allele.

Ji and colleagues sequenced the complete *vacA* gene from Ch2 and identified that it is highly similar to the m1 form of the VacA protein up to amino acid 648 and similar to the m2 form from amino acid 657 onwards (51). Vacuolation assays indicated that the chimeric toxin had the m1 phenotype and therefore the region from amino acid 657 onwards has no impact on the phenotypic differences between m1 and m2 strains (51). The distal *vacA* m region of the Kwäday Dän Ts'ìnchi *H. pylori* strain was typed as m1d. The

region that was sequenced began at amino acid 607 based on the Shi470 strain. It was not possible to determine the phenotype of the hybrid m region.

Yamaoka and colleagues analyzed the *vacA* region of 1042 *H. pylori* strains and identified one Japanese strain (JapanK1) with a combination of *vacA* m1b and m2 genotypes (21). Pan and colleagues undertook a study of the association between the *vacA* genotype of modern Chinese isolates and the resulting clinical outcome (19). In the course of their analysis, they identified four isolates with hybrid m1/m2 *vacA* genes with m1b proximal and m2 distal regions. Higher levels of vacuolating cytotoxin were noted in these hybrids as compared to isolates with only an m2 allele.

A study of 119 modern Taiwanese isolates identified 104 m2 strains, 13 m1 strains and two hybrid m1/m2 strains (53). The two strains were highly homologous to m1 strains in the region corresponding to 2701-2810 of U05676, but the analysis identified a greater homology to m2 strains in the region corresponding to 2540 – 2640 bp of Tx30a. Overall, *vacA* m region hybrids are rare (Table 3.3). Scientists have suggested that modern hybrids arose from a recombination between *vacA* m1 and m2 alleles during a mixed infection (52).

Table 3.3. Modern *H. pylori* hybrid *vacA* m1/m2 strains

Number of isolates	Geographical location	Source
4	China	19
2	Taiwan	53
1	China	49
1	Brazil	54
1	Japan	21

There is a possibility that the Kwäday Dän Ts'ìnchi individual was infected with more than one *H. pylori* strain, which would explain a *vacA* m hybrid region. Studies on modern *H. pylori* strains obtained from 65 children with and without duodenal ulcers revealed that ten children were infected with more than one *H. pylori* strain. One m1/m2 hybrid strain was identified (54). Modern *H. pylori* strains from 20 patients in Mexico City were characterized based on *vacA* and *cagA* types, and 17 individuals were identified as having an infection with two or more strains. Seven patients had untypeable middle regions and five patients had s2/m1 strains. The authors concluded that co-infection with multiple *H. pylori* strains was common in Mexico, and these strains appeared to have more diversity than those associated with other populations (55). Unfortunately, the complete *vacA* m region of the Kwäday Dän Ts'ìnchi *H. pylori* strain could not be sequenced. A breakpoint was not determined, but the strain was likely a *vacA* m hybrid because of the clean forward and reverse sequencing runs of the two *vacA* m regions.

3.4.1.4 *H. pylori vacA* s region

The Kwäday Dän Ts'ìnchi *H. pylori* strain was characterized as s2 in the signal sequence region of *vacA*. This finding suggests that the individual did not have major symptoms as a result of the *H. pylori* infection, and this is consistent with the negative pathology report. Previous studies have shown that toxigenic type s1 strains encode a protein that has a hydrophobic N-terminal region that can insert into lipid bilayers, whereas this region in non-toxigenic s2 strains contains a hydrophilic N-terminal extension that blocks vacuolating activity (17). Interestingly, the researchers identified that s2/m2 VacA

is capable of vacuolating activity if the N-terminal extension is removed. It is not understood why some *H. pylori* strains have a blocked capability of vacuolation.

A phylogenetic analysis of the *vacA* s region of the Kwäday Dän Ts'ìnchi *H. pylori* strain indicated similarities with modern strains that were analyzed by researchers studying the presence of *H. pylori* in the New World before Columbus (21). They characterized 1042 strains based on the *vacA*, *cagA* and *cag* right end junction genotypes. Most of the East Asian *H. pylori* strains were *vacA* s1c (94.7%), and none of the strains were s2 (21). They identified novel Native American *H. pylori* strains with an s1 subtype. Some of the strains (Colombia-NA1692, Alaska-2 and Alaska-7) clustered close to s1c, yet phylogenetic analyses of the *vacA* s region indicated that sequence differences between established subtypes was very low (for example between s1c and s2) (21). The Kwäday Dän Ts'ìnchi s2 sequence is highly similar to the *vacA* s sequences of two modern Alaskan *H. pylori* strains, Alaska-8 and -14 in the Yamaoka et al. study (21). Interestingly, these Alaskan strains were typed as m2a in the *vacA* m region with sequences similar to the m2a region in the Kwäday Dän Ts'ìnchi *H. pylori* strain.

The s2 subtype is not associated with Asian strains. *H. pylori* isolates were analyzed by Ghose and colleagues from patients located in Caracas, Venezuela and a center in the Venezuelan Amazon known as Puerto Ayacucho (34). The individuals from Caracas had European or mixed ancestry whereas the individuals from Puerto Ayacucho were of Amerindian ancestry. The samples from Caracas were identified with either *vacA* s1b or s2 subtypes whereas half of the Puerto Ayacucho isolates had *vacA* s1c subtypes, which has been identified in East Asian strains (34). Interestingly, 1 of 17 Puerto Ayacucho samples was characterized with an s2 subtype. The researchers suggested that their findings

indicate non-indigenous genes have been introduced into Puerto Ayacucho. The identification of an s2 region in the Kwäday Dän Ts'ìnchi *H. pylori* strain suggests that European strains were present in northern British Columbia prior to his lifetime.

Inserts within the s region of the *vacA* gene were analyzed in the Owen and Xerry study (48) that involved 484 modern strains from 32 countries. The short inserts (27 bp) found in s2 strains were highly conserved, and no connection with geographic origin was identified. Even with numerous polymorphisms, most (98%) of the s region inserts contained the NDPIHSESR amino acid sequence. The analysis of the Kwäday Dän Ts'ìnchi *H. pylori* strain revealed that the same conserved amino acid sequence was present. Owen and Xerry also identified that most s2 sequences contained a pre-insert motif (MGTELGANTP) in the s region (SRP type I) before the insert site. Five other SRP types were defined including M (1) to I or G (2) to S substitutions found in 10% of the strains. The Kwäday Dän Ts'ìnchi *H. pylori* s region contained a pre-insert motif IGTELGANTP.

During an analysis of isolates from 16 South African patients, a strain was identified with a *vacA* s2/m1 genotype. This finding was significant because it was one of the first strains to be typed with that particular *vacA* combination. Since all *vacA* combinations have been identified, this indicates that recombination events take place in *H. pylori* strains in vivo (56). A similar study in Mexico also identified s2/m1 strains (55). Further studies by Letley and colleagues involved the construction of a s2/m1 structure in both toxigenic and non-toxigenic backgrounds, and they found no difference with other strains under laboratory conditions (17). They were unable to clarify the reason for the smaller frequency of this particular *vacA* combination. The combination of the Kwäday

Dän Ts'ìnchi *H. pylori* s2 with m1d in the distal section of the middle region is significant because of the small number of modern *vacA* s2/m1 strains.

3.4.2 *H. pylori cagA*

The *cagA* gene is part of a pathogenicity island (PAI) that is 40-kb and contains 31 genes. Approximately 50 to 60% of *H. pylori* strains possess *cagA*, which is associated with duodenal ulcers and gastric cancer (45). Human hosts infected with *cagA* positive strains have evidence of more inflammation and higher levels of IL-8 (57) which is produced by macrophages to induce chemotaxis. Researchers have analyzed the association between the 3' end of the *cagA* gene and the geographical location of the infected human population. They identified that East Asian and non-East Asian strains can be separated based on this one variable region (57).

Studies by Zhou and colleagues indicated connections between *cagA* and human population movement (58). They examined 143 East Asian isolates (Fukui and Okinawa, Japan and Hangzhou, China) obtained from patients with duodenal ulcers or chronic gastritis. They identified that the presence of *cagA* positive and Western type CagA strains was significantly different between Fukui and Okinawa. Sequence differences in the *cagA* positive strains indicated that while most had the East Asian type CagA, 21.7% of the strains in Okinawa had a Western type CagA, which may be an indication of the influence of an international connection with the West.

In this study, the *cagA* status of the Kwäday Dän Ts'ìnchi *H. pylori* strain was not determined due to lack of amplification in that region. Unfortunately, no *cagA* PCR products were obtained when previously published primers and primers specifically developed for this study were used (45, 59). Since a false negative result may be due to

DNA degradation, further steps were taken to determine if the region around the *cagA* PAI could be identified. The *cag* PAI is flanked by 39 bp direct DNA repeats, and *H. pylori* strains that are *cagA* negative do not have a complete PAI, but they do possess a single copy of the 39 bp sequence in the glutamate racemase gene. Partial *cag* islands and size variation have been identified (60). Unfortunately, I was unable to confirm that the *H. pylori* strain was *cagA* negative because no DNA amplification occurred with primers that flanked the direct repeat region.

A statistical connection has been determined between the *vacA* s1 genotype and the presence of the *cag* PAI (60), and studies have shown that *vacA* s2 strains are associated with the lack of a *cag* PAI even though the two sites are far apart on the *H. pylori* chromosome. Andreson and colleagues isolated and amplified the *vacA* and *cagA* regions in 156 *H. pylori* strains from patients in Estonia (61). They identified an s1a region in 135 of the strains whereas 16 strains were characterized as s2. Interestingly, 15 of the 16 s2 *H. pylori* strains were also *cagA* negative. There is a possibility that the Kwäday Dän Ts'ìnchi *H. pylori* strain is *cagA* negative because of the potential correlation with a *vacA* s2 genotype.

3.4.3 *H. pylori flaA*

Motility of *H. pylori* is influenced by the expression of the *flaA* gene. The bacterium has multiple flagella that consist of a basal body along with a central filament covered by a membranous sheath. Flagellin A is responsible for the central filament which is required for motility. The flagella enable *H. pylori* to swim to regions of higher pH below the mucosal surface (9). The gene *flaA* is highly conserved and researchers have

determined that it is a major antigen that results in the production of antibodies (62). The *flaA* amino acid sequence of the Kwäday Dän Ts'ìnchi strain differs by only one amino acid (lysine) from Shi470 (arginine) (Table 3.4). Lysine has been identified in that position in other *H. pylori* strains.

H. pylori Shi470 is an isolate from an Amerindian resident in Shimaa, Peru, which is a remote Amazonian village. The complete genome was recently sequenced, and the information at this date is only available online. Analyses of the Shi470 *H. pylori* strain indicated that it is more closely related to strains from East Asia and thought to represent a Native American strain prior to the arrival of Europeans (15).

It is noteworthy to mention that Shi470 also shares a high similarity (95%) in the distal *vacA* m region with the Kwäday Dän Ts'ìnchi strain, which I have typed as m1d according to Yamaoka et al. study (21). The similarities in these two regions are intriguing because of the link between *H. pylori* and different human populations. The modern Shi470 strain and the ancient Kwäday Dän Ts'ìnchi strain were associated with individuals who likely had ancestors from the same population thousands of years previously.

Table 3.4. FlaA amino acid sequence comparison between the Kwäday Dän Ts'ìnchi *H. pylori* strain and the Shi470 strain

KDT FlaA
QVDGVNDVTLESVKISSSAGTGIGVLAEVINKNSNQTGV***K***AYANVITTSDVAVQSGSLSNL
TLNGIHLGNIADIKKNDSDGRLV
Shi470 FlaA
QVDGVNDVTLESVKISSSAGTGIGVLAEVINKNSNQTGV***R***AYANVITTSDVAVQSGSLSNL
TLNGIHLGNIADIKKNDSDGRLV

3.4.4 The impact of modern *H. pylori* infections in Northern Aboriginal communities

In this modern era, humans are now experiencing everything at a global level, especially disease. Air travel has enabled people and pathogens to visit different environments in record time. Epidemiologists will continue to face challenges ahead while tracking the sources of various disease-causing organisms. Scientists are now recognizing the importance of understanding the genetics of the human host populations, since it is apparent that these genetic differences are influencing the impact of the same pathogen on different populations.

Studies of the presence of *Helicobacter pylori* in modern Alaskan Aboriginal populations living in Norton Sound have shown that infection rates are high (80% of the 610 individuals were seropositive for *H. pylori* antibodies and 72% were seropositive by 24 years of age) (63). Many Alaskan *H. pylori* isolates have been found to be resistant to clarithromycin or metronidazole (64).

It has been postulated that *H. pylori* plays a role in the iron deficiency anemia that has been identified in the Alaskan Aboriginal population (65). Anemia was found to remain high even following iron supplementation suggesting that it was a result of an infection (10). In 2006, Canadian Aboriginal communities were identified by a Canadian *Helicobacter* Study Group as a population at most risk of developing a *Helicobacter*-related disease (10). Studies in the circumpolar region also identified high levels of *H. pylori* infections in the communities of Greenland and Russia.

This study identified that a *vacA* s2-m2a/m1d *H. pylori* strain was associated with Kwäday Dän Ts'ìnchi. It is likely that the individual did not have any symptoms as a result of the *H. pylori* infection because the strain was a non-toxigenic *vacA* s2, which

corresponds with the negative pathology results. Based on the identification of a hybrid region in the ancient *H. pylori* strain (*vacA* m2a/m1d), and due to the current high rates of infection in the circumpolar region, further studies need to include the identification of complete *vacA* m sequences in the modern *H. pylori* strains isolated from individuals living in Northern communities to gain a better understanding of the role that the *vacA* m region plays in the virulence of the bacterium in that locality.

3.5 CONCLUSIONS

In this study, *H. pylori* DNA was amplified and sequenced from the stomach tissue of the Kwäday Dän Ts'ìnchi ancient individual who was recovered from a glacier in northern British Columbia, Canada. Through an analysis of the *H. pylori vacA* gene, a hybrid m2a/m1d allele and an s2 signal region were identified. A portion of the *flaA* gene was also amplified and sequenced. The amplification of a *cagA* gene fragment was unsuccessful, but the strain may not be *cagA* negative because of possible DNA damage in that region.

Modern infection rates of Aboriginal individuals in the circumpolar regions are high and some treatments have been unsuccessful due to antibiotic resistance. The strain associated with the Kwäday Dän Ts'ìnchi individual carried a *vacA* s2 allele, which is an indication that the strain was non-toxigenic. The presence of an s2 allele, which is unusual in Asian strains, suggests that European strains were present during the timeframe of AD 1670 to 1850.

The characterization of the *vacA* m region revealed a hybrid region that is rare in modern strains. The phylogenetic analysis indicated that the m1d region clustered with

previously studied novel Native American strains that were closely related to Asian strains. These observations are consistent with the idea that the first humans who migrated into the New World crossed over the Bering Strait from Asia.

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CHAPTER FOUR:

The Discovery of a Latent *Mycobacterium tuberculosis* Infection in Ancient Human Remains Recovered from a Northern Canadian Glacier

ABSTRACT

Mycobacterium tuberculosis is a bacterial pathogen responsible for the disease tuberculosis that has plagued humanity throughout history. Even with current medical knowledge, one in three individuals worldwide is infected. Ten percent of these individuals will become ill, and currently this disease results in two million deaths per year. The analysis of ancient microbial DNA associated with past human infections is useful in defining the spatial and temporal associations between humans and their pathogens. In 1999, an ancient individual was recovered from a glacier in northern British Columbia, Canada. Radiocarbon dates indicated a timeframe of AD 1670 to 1850, which is either precontact or early European contact for that area. The Aboriginal elders named the site *Kwäday Dän Ts'ìnchi* and gave permission for multiple research projects to learn more about their ancestor. This study began with the testing of Kwäday Dän Ts'ìnchi tissue for the presence of microbial DNA associated with *Mycobacterium tuberculosis.* Even though a pathological and histological examination was negative, mycobacterial DNA was positively identified in the lung tissue with primers specific for the IS*6100* insertion sequence. The ancient TB strain was characterized through genetic analyses involving the *gyrB* and *katG* regions. The presence

of a single nucleotide polymorphism (G) at position 1388 of the *katG* gene places the strain in genotype Lineage II, which is more commonly associated with European *M. tuberculosis* strains. Evidence of two *gyrB* alleles that differed by one amino acid was identified. This suggests a selective pressure even though it was a time prior to the use of antibiotics. A previous study identified a large quantity of *Salicornia perennis* (glasswort) in a stomach sample, which the elder's suggested was either part of a traveller's diet or a medicine. Recently a patent was granted to a group of scientists who identified the antitubercular properties of *Salicornia brachiata* extract. A natural compound in the plant may have resulted in the selection of a mutation in the *gyrB* gene of the ancient *M. tuberculosis* strain.

4.1 INTRODUCTION

Tuberculosis (TB) is a devastating disease world-wide despite advances in chemotherapeutic treatments (1). It can be acute or chronic and impact both soft and skeletal tissues. The disease is the result of an infection by a member of the *Mycobacterium tuberculosis* complex of bacteria. This complex consists of the human pathogens *M. tuberculosis, M. africanum,* and *M. canettii*. *M. bovis, M. microti and M. caprae* are also members that are more often associated with animal infections. *M. canettii* is the most genetically diverse of the group and is currently considered to resemble the progenitor strain of the complex (2).

Transmission mainly occurs as a result of inhaling the bacteria into the lungs where replication occurs within the macrophages of the host's immune system. *M. bovis* may also be transmitted through ingestion of infected food or milk (3). *M. tuberculosis* is not found

in the environment and the only natural reservoir is humans. Every year approximately 8 to 10 million people develop active tuberculosis, and it was the cause of 1.7 million deaths in 2006 (4).

According to the World Health Organization, one third of the world's population has a latent tuberculosis infection of which five to ten percent will eventually develop active TB. Individuals with a latent TB infection have no symptoms and are not infectious to others but they may develop active TB during their lifetime. Activation of a latent tuberculosis infection is often the result of a disruption in the balance of an individual's immune system. Stress, malnutrition, host genetics, and infection with other micro-organisms, such as HIV, are some factors that affect the outcome of a latent infection or make individuals more susceptible to new infections (5).

The first complete genome sequence of a well-characterized *M. tuberculosis* strain H37Rv was published in 1998 (6). The size of the genome is 4,411,529 base pairs with approximately 4000 genes. Since the first *M. tuberculosis* genome was sequenced, the genomes of strains CDC1551, F11 and H37Ra have also been sequenced (7, 8) which has enabled scientists to complete detailed comparisons. This new data has resulted in a clearer understanding of the evolutionary relationships between the strains, and this has been used to study population movement. It also offers new tools for the identification and characterization of ancient strains.

4.1.1 Phylogenetic analysis of modern *M. tuberculosis* strains indicate a connection with past human migration

Numerous genotyping methods have helped to define phylogenetic relationships. Barnes and colleagues reviewed some of the most common genotyping methods used on modern *M. tuberculosis* strains (9). Restriction fragment length polymorphism (RFLP) analysis of the insertion sequence IS*6110* is one method. The insertion sequence varies in copy number and distribution between the *M. tuberculosis* strains. Another genotyping method involves the determination of the number and size of 12 MIRUs (mycobacterial interspersed repeat units) that have two to eight alleles at each locus. MIRUs are regions within the genome that contain repetitive sequences of varying lengths. Spoligotyping (spacer oligonucleotide typing) can also be used to differentiate between *M. tuberculosis* strains. A direct repeat locus within *M. tuberculosis* contains multiple copies of a 36 bp direct repeat. These copies are separated by spacers with different sequences and since these sequences are conserved, the analysis of the presence or absence of these spacers is used in the determination of strain differences.

As additional sequence data is acquired through rapidly evolving technology, more information is available to investigators who are piecing together the evolutionary history of the bacterium *M. tuberculosis*. A recent study by Gutierrez and colleagues determined that even though low levels of genetic variation identified between modern *M. tuberculosis* isolates suggest the bacterium went through an evolutionary bottleneck, a progenitor species may have been contemporaneous with early hominids in East Africa (2).

With the goal of determining the timing of divergence, the diversity of the populations and the spread of the bacteria, Wirth and colleagues analyzed 24 MIRU loci in

355 isolates that were chosen to represent a global distribution (10). They determined that a radiation of two lineages emerged from an East African progenitor approximately 40,000 years ago followed by movement of the bacterium into Asia, Europe and Africa. This is compatible with human migration studies that suggest modern humans migrated out of Africa. Interestingly, the study showed that the lineages had a recent expansion around 180 years ago at a time that coincides with the industrial revolution and human population expansion.

Neutral genetic variation in *M. tuberculosis* genes associated with antimicrobial drug resistance was analyzed in a phylogenetic study by Baker and colleagues (11). They identified silent single nucleotide polymorphisms (SNPs) in seven gene loci from 316 clinical isolates and determined evolutionary connections between four distinct *M. tuberculosis* lineages with geographic associations. The lineages were designated I through IV with associations between lineage I and Southeast Asia, lineage II and Europe, and lineage III and India. Lineage IV was identified with a global distribution.

In a study of the global *M. tuberculosis* population structure, Gagneux and colleagues defined six phylogeographical lineages (12). Through the analysis of large sequence polymorphisms, the study attempted to address the impact of the lengthy connection between this pathogen and its human host. They determined that in urban settings, the spread of a particular bacterial lineage occurred in sympatric populations, those which occupy the same geographic location, whereas if an allopatric transmission occurred, it was likely due to impaired host resistance in a high-risk individual. This expands on previous findings that the host's region of origin is predictive of the strain, and this association is seen even when the transmission occurs outside the region of origin (13).

Gutacker and colleagues analyzed 36 synonymous SNPs in 5069 *M. tuberculosis* strains isolated from American and European patients (14). Since a synonymous polymorphism does not result in an amino acid change, they are considered to be evolutionary neutral. The strains in the study were assigned to one of nine major genetic clusters. Because of unequal distribution, the clusters indicated geographic subdivisions. This is further evidence of the connection between the bacterium and the larger scale host population.

In a recent study, 212 SNPs were analyzed in a collection of modern *M. tuberculosis* strains. The investigators identified genetic diversity that resulted in the creation of six phylogenetically different cluster groups. The clusters were associated with the geographical origin of the strains as well as the birthplace of the host. The study indicated that the oldest clusters were associated with India followed by East Asia. Less diversity was identified in Mexican indigenous populations which led the authors to suggest that the bacterium had a more recent introduction into the area (15).

Hershberg and colleagues analyzed the genetic diversity within the *M. tuberculosis* complex with seven megabases of sequence data from 108 modern strains that were selected to represent global diversity (16). Their analysis indicated that members of the *M. tuberculosis* complex are more genetically diverse than what was originally assumed based on previous studies. They concluded that genetic drift has played a role in the diversity of the *M. tuberculosis* complex, which is connected to both past and present human migration.

Phylogenetic analyses for migration studies typically use modern *M. tuberculosis* isolates, but the recently developed field of paleomicrobiology also incorporates sequence data from the genetic analysis of ancient micro-organisms to define evolutionary

relationships. Researchers have successfully identified past *M. tuberculosis* infections using the knowledge from modern strains, especially the presence of the IS*6110* insertion sequence (17). Unfortunately, the use of most modern genotyping methods on ancient strain sequences is problematic due to the requirement of lengthy intact sequence information.

Ancient sequences are often degraded by post-mortem oxidation and hydrolysis. The amount of DNA degradation is dependent on the environmental conditions, with cold and dry locations the most ideal for DNA preservation. Ancient DNA analysis involves the amplification of small regions of DNA often smaller than 200 bp. This limits the range of genotyping methods, but the identification of SNPs has potential for not only genotyping modern strains but ancient *M. tuberculosis* strains as well.

4.1.2 The identification of ancient tuberculosis through skeletal and molecular diagnoses

Before sequence information from ancient *M. tuberculosis* strains can be analyzed for the evolutionary relationships associated with human migration, it is necessary to first identify likely sources of the genetic material in an archaeological context. In 1993, Spigelman and Lemma reported the first diagnosis of an *M. tuberculosis* complex infection in ancient remains with paleopathologies representative of the disease tuberculosis (18). The positive molecular identification of a pathological ulna from pre-European contact Borneo was noteworthy. A few years later, concerns were raised regarding the methodology used since the field of ancient DNA research was still very young (19). The scientists revisited their earlier research and confirmed the original findings (19).

In 1997, Nerlich and colleagues reported molecular evidence of an *M. tuberculosis* infection in an Egyptian mummy dated from the New Kingdom (1550 – 1080 BC) (20). The male mummy was recovered at the Tombs of the Nobles in Thebes-West, Upper Egypt. Upon visual inspection, extensive pleural adhesions to the chest wall were identified in addition to anterior destruction of two lumbar vertebrae. These pathologies are indicative of the disease tuberculosis, and the ancient mycobacterial infection was confirmed using molecular technology (20).

Members of the *M. tuberculosis* complex are unique because their cell walls contain a high concentration of lipids. Postmortem DNA does not survive indefinitely mainly due to oxidation and hydrolysis, but *M. tuberculosis* complex DNA is more likely to remain intact. Researchers have suggested that the lipid mycobacterial cell wall protects the bacterium from postmortem enzymatic attack (17). Also, *M. tuberculosis* complex DNA contains a high percentage of guanine and cytosine that aids in DNA stabilization (17).

Numerous studies have now indicated the presence of *M. tuberculosis* complex DNA in ancient mummified and skeletal tissues (21). The capability of legitimately diagnosing ancient tuberculosis through molecular technology has been an exciting addition to the field of paleopathology. Molecular methods can now be applied to answer archaeological questions including: when did tuberculosis arrive in the New World and by what means? How can this knowledge then translate to understanding the current global transmission of the disease? These questions will be discussed below.

4.1.3 Precontact tuberculosis in the Americas

For many years, it was assumed that tuberculosis was brought to the New World by Europeans post 1492. Pre-European contact osteological evidence of the disease, including anterior vertebral destruction, has been identified in skeletal material from numerous archaeological sites (22). Scientists were skeptical of the TB diagnoses given to these remains mainly because of the devastation that the disease caused to the indigenous people when they first encountered Europeans, suggesting that the population had not been previously exposed (23).

4.1.3.1 South American histological and molecular evidence of precontact TB

In 1973, Allison and colleagues reported their findings of a tuberculosis case from precontact Peru (AD 700) (24). The TB diagnosis was based on many pieces of evidence including: an anterior curvature of the spine due to the destruction of vertebrae (Pott's disease), a psoas abscess, and lesions in the lungs, liver and right kidney. They also identified through staining techniques the presence of acid-fast organisms, which is a characteristic feature of the bacterium *M. tuberculosis*.

Salo and colleagues reported the identification of *M. tuberculosis* DNA in a pre-Columbian Peruvian mummy (25). This was the first occasion that a DNA analysis was performed to identify a pre-European contact TB case. The mycobacterial DNA was extracted from a lesion in the upper right lobe of the lung in a spontaneously mummified adult female with a radiocarbon date of 1040 ± 44 years BP. The following year, Arriaz and colleagues identified mycobacterial DNA in a vertebral lesion from a Chilean

mummified child with a radiocarbon date of 910 ± 70 years BP and skeletal evidence of Pott's disease (26).

1.3.2 North American skeletal and molecular evidence of precontact TB

Gomez i Prat and colleagues summarized the numerous cases of North American precontact tuberculosis (28). All diagnoses were based on osteological evidence, and only one individual from the Joe Gay site in Illinois was found with soft tissue that suggested a pleural lesion. Most of the North American TB pathologies were associated with remains recovered in the Central Plains of the USA and around Lake Ontario in Canada. The oldest North American precontact TB diagnosis was made in 1886 by Dr. William F. Whitney of the Harvard Medical School on skeletal remains from Nashville, Tennessee, which were identified with an extreme anterior curvature and fusion of numerous vertebrae (23).

Skeletal evidence of precontact tuberculosis is limited in Canada perhaps due to different burial practices of the early cultures and limited disturbance of burial sites by modern researchers. One precontact case was reported in western Canada based on the identification of ankylosed first and second lumbar vertebrae with loculated cavities. This evidence was associated with an adult female approximately 45 yrs at death with a radiocarbon date of 975 ± 75 BP. The skeletal remains were accidentally discovered during quarry operations in southeast Saskatchewan (Woodlawn Site) (29).

At this time, only two North American samples have been positively identified for the presence of *M. tuberculosis* complex DNA (30). The two samples included fused lumbar vertebrae with a radiocarbon date of 460 ± 80 BP from the Uxbridge Ossuary near Toronto, Canada and a thoracic vertebra from an early 11th century Middle Mississippian

burial (Schild Cemetery in Illinois). Both samples had skeletal lesions indicative of the disease TB. An earlier analysis of the Uxbridge Ossuary revealed numerous examples of lytic lesions in lower vertebral and sacro-iliac area. These observations were regarded as evidence for the presence of skeletal tuberculosis (31).

Interestingly, *M. tuberculosis* complex DNA was identified in the metacarpal of an extinct long-horned bison radiocarbon dated 17,870 ± 230 BP (32). Skeletal material from this Pleistocene bovid was recovered in Natural Trap Cave, Wyoming and the appearance of an undermined articular surface of the metacarpal was suggestive of the disease tuberculosis. This discovery of mycobacterial DNA is important because it indicates that *M. tuberculosis* has been present in the New World for millennia.

4.1.4 Evidence of the antiquity of tuberculosis on the Canadian Northwest Coast

Evidence of precontact tuberculosis on the Northwest Coast of Canada has yet to be identified, but ancient human remains recovered from that region were found with preserved tissue suggestive of a TB infection. In the 1970s, the remains of a female recovered from St. Lawrence Island, Alaska (AD 400) were autopsied, and a calcified lymph node was discovered. The tissue was negative for the presence of acid-fast bacilli when specific stains were used (33). This negative finding is inconclusive because these stains have not been consistent on ancient tissue infected with mycobacterial DNA (34). Another female (AD 1500) was recovered near Barrow, Alaska, and calcified granulomas in the right lung and lymph nodes near the main bronchi were identified. Both cases were originally thought to be the result of histoplasmosis (a fungal disease), because it was assumed at the time that tuberculosis was a post-contact disease (35).

Historical records indicate that the first documented case of tuberculosis in a Northwest Coast Aboriginal occurred in 1793 at Nootka Sound on Vancouver Island, where the Spanish had established a post that was occupied between 1790 and 1795. Tuberculosis eventually affected the local communities near modern day Sitka, which had been occupied by the Russians since 1799 (36). During the 1840s, the Russian-American Company known for dealing with furs had their headquarters in Sitka. The company physicians maintained medical records that included comments on the presence of inflammatory diseases such as pleurosis, pneumonia and "blood-spitting" during the winter months (37).

The first osteological evidence of tuberculosis on the Northwest Coast corresponds with a historic case identified in a Barkley Sound burial cave on Vancouver Island. During an assessment of the cave in Nuu-chah-nulth territory, numerous fused thoracic vertebrae were discovered with evidence of an anterior collapse from the third to the sixth thoracic vertebrae. There was no visible reactive bone growth and no involvement of the neural arch or spinous processes, which led the investigators to conclude that this was likely a case of tuberculosis (38).

4.1.5 The identification and characterization of an ancient *M. tuberculosis* strain associated with the Kwäday Dän Ts'ìnchi ancient individual

In August 1999, three hunters discovered frozen human remains in a glacier in Tatshenshini-Alsek Park in northern British Columbia on the traditional territory of the Champagne and Aishihik First Nations (Figure 4.1). An agreement was made between the provincial government and the Champagne and Aishihik First Nations to allow for a scientific analysis of the individual from the site named Kwäday Dän Ts'ìnchi by the

Aboriginal elders. Initial radiocarbon dates suggested the individual was approximately 550 years old (AD 1450), which was a time before the arrival of Europeans into the area (39). Recently, further radiocarbon analyses were performed and the researchers discovered that the individual was from a more recent timeframe of approximately AD 1670 to 1850 (40). This is still either precontact or early contact for that region. A pathological and histological examination did not reveal a cause of death, and there was no evidence of illness (39).

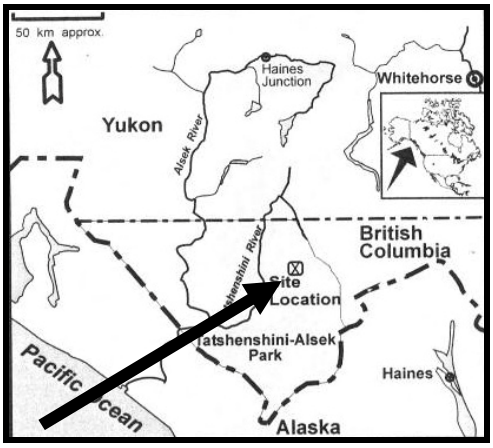

Figure 4.1. Kwäday Dän Ts'ìnchi site (40)

As part of an ancient microbial DNA study, the tissues of the Kwäday Dän Ts'ìnchi ancient individual were tested for evidence of *M. tuberculosis* complex bacteria. In this paper, the finding of *M. tuberculosis* DNA will be discussed, along with the details of characterization of the ancient *M. tuberculosis* strain. The significance of the identification of a latent tuberculosis infection and the relevance of the modern phylogenetic data on ancient human migration will be reviewed. Finally, a simple method will be suggested to differentiate between *M. tuberculosis* and *M. bovis* infections through the identification of differences in the *Rv3479* gene previously identified in the sequenced genomes of modern *M. tuberculosis* complex strains.

4.2 METHODS

4.2.1 Sample Collection

Small tissue samples (approximately half a gram or less of lung, liver, myocardium, lymph node, spleen, stomach, small intestine, cecum, descending colon, skin and rib tissues) were retrieved from the ancient individual during an autopsy at the Royal British Columbia Museum in Victoria, British Columbia. Standard protocols were followed to prevent contamination. All members of the autopsy team were dressed in appropriate protective clothing, and sterile surgical tools were used. The samples were packed on ice in an insulated container for travel to the University of Saskatchewan, in Saskatoon, Saskatchewan, where they were stored in a -70°C freezer upon arrival.

4.2.2 DNA Extraction

Tissue extractions were set up in a separate laboratory in a biological safety cabinet that was surface cleaned with 10% (v/v) Clorox bleach and NucleoClean Decontamination wipes (Millipore, MA). DNA was extracted using a silica-based method suggested for the recovery of ancient DNA by Yang and colleagues (41) with spin columns from a QIAquick® PCR Purification Kit (QIAGEN Inc., Mississauga, Ontario).

4.2.3 PCR Amplification

All PCR reactions were set-up in a separate location from the post-PCR laboratory. The components of a 50µl reaction consisted of: 5 µl DNA extract, 2 Units of AmpliTaq® Gold DNA Polymerase (Applied Biosystems Canada, Streetsville, Ontario), GeneAmp® PCR Gold Buffer (15mM Tris-HCl, pH 8.0 and 50mM KCl), 2.5 mM $MgCl_2$, 200µM each

dNTP from GeneAmp® dNTP Mix (Applied Biosystems Canada, Streetsville, Ontario), and previously published and newly designed PCR primers (0.2µM Sigma Genosys Canada, ON) (Table 4.1). PCR reactions were performed in an MJ Mini Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) as follows: 12 minutes initially at 95˚C, followed by 40-45 cycles of denaturation at 95˚C for 1 min, differing annealing temperatures for 1 min followed by 72˚C for 1 min. After the cycles were completed, the temperature was held at 72˚C for 8 minutes.

4.2.4 DNA Electrophoresis

Five microlitres of each amplification product were added to individual wells in 2% (w/v) agarose minigels that were immersed in a Tris/acetate/EDTA (1xTAE) solution. After the application of 88 volts for one hour, gels were removed and placed in a container containing a 0.5 µg/ml ethidium bromide solution for 10 minutes. The gels were destained in water for 10 minutes before visualization on a UV lightbox (254 nm).

4.2.5 DNA Sequencing

PCR products were sequenced directly in both forward and reverse directions on an ABI 3730*xl* DNA sequence analyzer at the National Research Council-Plant Biotechnology Institute (Saskatoon, Saskatchewan). The DNA sequences were compared with reference sequences in the National Institute of Health (NIH) Genbank database.

TABLE 4.1: PCR primers for the amplification of regions within *M. tuberculosis*

Region	Primers		Product size	Annealing Temp. Number of Cycles	Source
IS*6100*	P1	CTCGTCCAGCGCCGCTTCGG	123bp	68˚C	43
	P2	CCTGCGAGCGTAGGCGTCGG		40 cycles	
Rv3479	MTB1	ATGTGTAGCAGACCAGCGAT	156bp	62˚C	This study
	MTB2	GGCAAGTTGCGTCAAGGT		40 cycles	
TbD1	TbD1iF	AAGGACGAAAGGATCGTCAA	135bp	62˚C	34
	TbD1iR	AAAACAGCAAGATCGGCAAC		40 cycles	
	TbD1fF	CGGTTATCGAAAGGCTAACG	97bp	62˚C	34
	TbD1fR	TACCGTCGATCGTGTCAAAG		40 cycles	
gyrB	TSgyrBF	CACATCAACCGCACCAAGAAC	203bp	64˚C	This study
	TSgyrBR	TTGTTCACCACCGACGTCAG		45 cycles	
	TSgyrB2F	ACACCATCAACACCCACGAG	209bp	64˚C	This study
	TSgyrB2R	CAACTTGGTCTTGGTCTGGC		45 cycles	
	TSgyrB3F	AGGTCAGCGAACCGCAGTTC	200bp	64˚C	This study
	TSgyrB3R	CACCAACTCTCGTGCCTTAC		45 cycles	
katG	katGF	TCAGCCACGACCTCGTCGG	163bp	68˚C	34
	katGR	AGGCGGATGCGACCACCGTT		45 cycles	

4.3 RESULTS

4.3.1 The positive identification of *M. tuberculosis* complex DNA

In this study, polymerase chain reaction (PCR) with previously published primers designed to amplify a 123 bp region of the IS*6100* insertion sequence specific for *M. tuberculosis* complex (43) was used on extracted Kwäday Dän Ts'ìnchi lung tissue (Figure 4.2). *M. tuberculosis* complex DNA was also amplified in multiple tissue samples from the thorax and abdomen including: a lymph node, spleen, liver, stomach, small intestine, cecum, and descending colon. A skin sample and rib sample were also tested, but the PCR was negative (Figure 4.3).

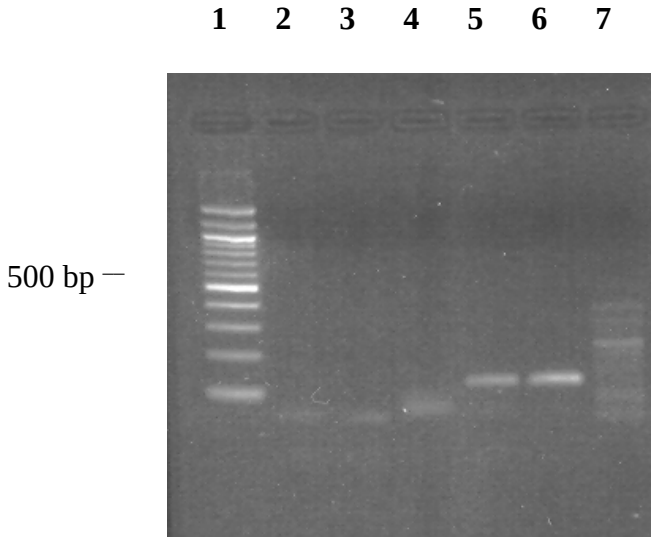

Figure 4.2. Amplified IS*6100* PCR products (123 bp) on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, lung sample #1 amplified with previously published IS*6100* primers P1 and P2 (43); **Lane 3**, negative extraction **Lane 4**, lung sample #2; **Lane 5**, positive lung sample #3; **Lane 6**, positive myocardium extract; **Lane 7**, negative PCR

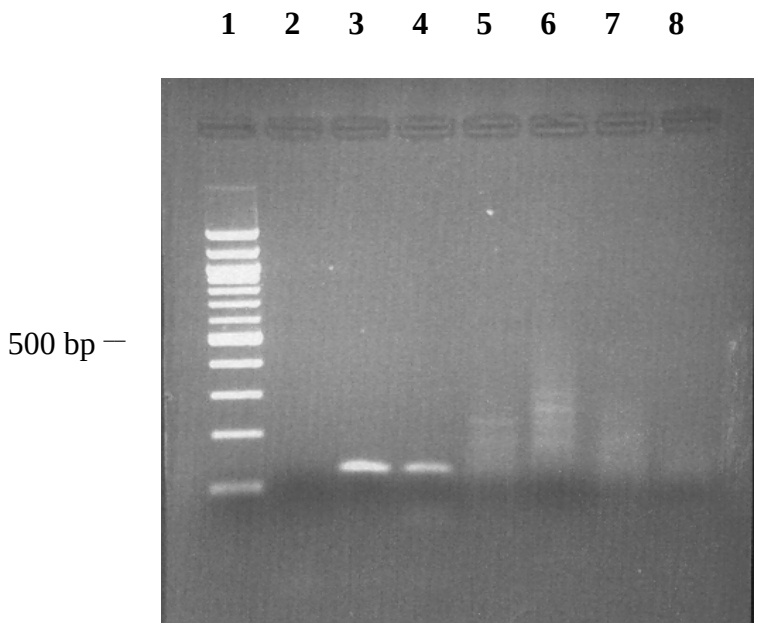

Figure 4.3. Amplified IS*6100* PCR products (123 bp) on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, Unconfirmed spleen tissue extract amplified with previously published IS*6100* primers P1 and P2 (43); **Lane 3**, Liver extract; **Lane 4**, Small intestine extract; **Lane 5**, Rib extract; **Lane 6**, Skin extract; **Lane 7**, Negative extraction; **Lane 8**, Negative PCR

One extraction blank was used to monitor for contamination. It was positive on occasion when IS*6100* primers were used but often negative when single gene analyses (*gyrB, katG*) were performed. This positive result indicated that there was a source of contamination. It is possible that the contamination may have occurred during the DNA extraction from the tissues. Moisture was noted on the spin columns after centrifugation, and DNA may have inadvertently been transferred from one sample to another through the handling of multiple extraction tubes while wearing the same pair of gloves. I subsequently ruled out any contamination in all laboratory items used and in all of the extraction

solutions. Lung tissue was sent to a second laboratory for confirmation of the positive identification of *M. tuberculosis* complex DNA. Dr. Mark Spigelman (The Hebrew University) reported that they also amplified and sequenced *M. tuberculosis* complex DNA.

4.3.2 Ruling out *Mycobacterium bovis*

The IS*6100* sequence identifies members of the *M. tuberculosis* complex but further characterization was necessary to classify the exact bacterial infection. The members of the complex that are most often associated human tuberculosis include *M. tuberculosis*, *M. bovis* and *M. africanum*. *M. africanum* is very rare in North America, but *M. bovis* has been identified in North American animals. Eating infected meat or drinking infected milk can result in the transmission of the bacterium to humans. Interestingly, only one case of an ancient *M. bovis* infection has been identified (44).

I developed a fast and simple way of ruling out *M. bovis* by analyzing a segment of the *Rv3479* gene, which is complete in *M. tuberculosis* but contains a deletion in *M. bovis*. Primers were designed that would amplify 156 bp of the *Rv3479* gene that exists in the *M. tuberculosis* genome but is deleted in *M. bovis*. The *Rv3479* gene sequence was successfully amplified from the tissue extract, therefore ruling out an *M. bovis* infection (Figure 4.4). Unfortunately the one negative extraction control indicated contamination, but as discussed above, the evidence of contamination was sporadic.

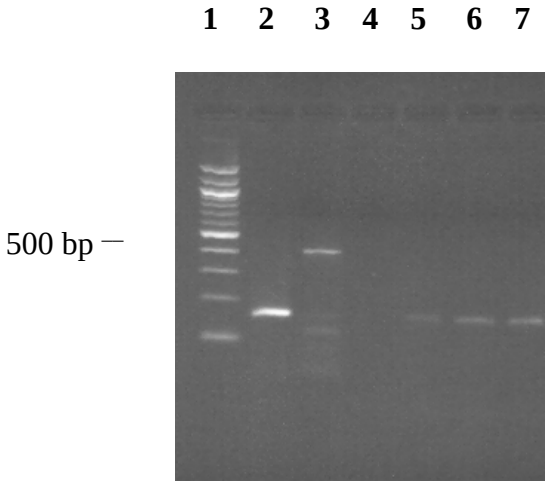

Figure 4.4. Amplified *Rv3479* PCR products (156 bp) on a 2% agarose gel (primers MTB1 and MTB2) **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, positive control (prepared in a different laboratory); **Lane 3**, *M. bovis*; **Lane 4**, negative PCR; **Lane 5**, liver extract; **Lane 6**, lung extract; **Lane 7**, negative extraction

4.3.2.1 TbD1

Tuberculosis has had a long association with humans. Modern *M. tuberculosis* isolates are either genetically 'modern' based on the appearance of an *M. tuberculosis* specific deleted region (TbD1) or they resemble an ancestral strain if the region is present (45). No amplification occurred when internal primers for the TbD1 deleted region were used, whereas 97 bp were successfully amplified using previously published primers that flank the region (34), indicating that the Kwaday Dan Ts'ìnchi *M. tuberculosis* strain is genetically modern (Figure 4.5).

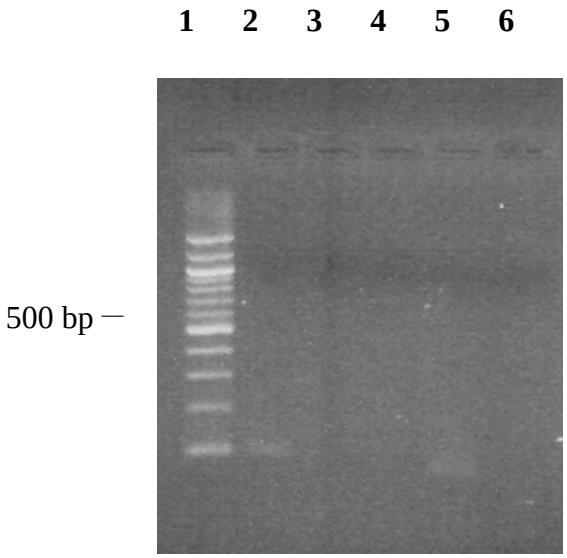

Figure 4.5. Amplified PCR products (97 bp) flanking the TbD1 region on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, small intestine extract positive amplification with TbD1fF and TbD1fR primers (34); **Lane 3**, lung extract; **Lane 4**, negative extraction; **Lane 5**, negative extraction; **Lane 6**, negative PCR

4.3.3 Single gene analyses

Genetic analyses of *M. tuberculosis* isolates have revealed that there is little sequence diversity with the exception of some of the repeat sequences, thus seemingly insignificant nucleotides changes are still important for phylogenetic studies (46). The identification of an *M. tuberculosis* infection by IS*6100*, *Rv*3479 and regions flanking the TbD1 region indicated the presence of the bacterium's DNA in Kwäday Dän Ts'ìnchi tissues but no additional information could be gathered from these regions. It was

important to perform additional single gene analyses that included the portions of the *gyrB* and *katG* genes.

4.3.3.1 *gyrB*

Three sets of overlapping primers were designed to study a region of the Kwäday Dän Ts'ìnchi *M. tuberculosis gyrB* gene. A total of 495 bp were amplified (Figure 4.6).

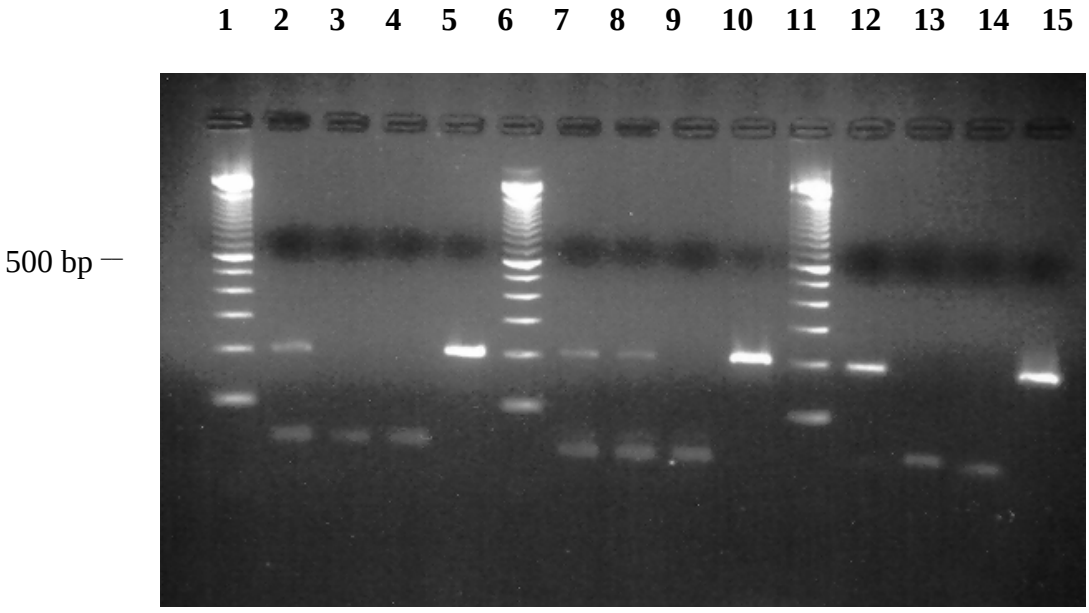

Figure 4.6 Amplified PCR products of the *gyrB* region on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, stomach extract amplified with primer set #1 (203 bp); **Lane 3**, negative extraction; **Lane 4**, negative PCR; **Lane 5**, H37Rv; **Lane 6**, 100 bp ladder; **Lane 7**, stomach extract amplified with primer set #2 (209 bp); **Lane 8**, negative extraction; **Lane 9**, negative PCR; **Lane 10**, H37Rv; **Lane 11,** 100 bp ladder**; Lane 12**, stomach extract amplified with primer set #3 (200 bp); **Lane 13**, negative extraction; **Lane 14**, negative PCR; **Lane 15**, H37Rv.

The Kwäday Dän Ts'ìnchi *M. tuberculosis gyrB* sequence indicated evidence of two alleles. One nucleotide position was identified to contain two bases in both the forward and reverse sequencing runs (Figure 4.7). A Blast search with the one 'allele' indicated that the sequence shared a 100% identity with one modern *M. tuberculosis* isolate from Japan KY679 (47), whereas the sequence of the second allele matched many sequences in the database . The Japanese strain KY679 and the one allele of the ancient Kwäday Dän Ts'ìnchi strain both have a C at position 1117, whereas the other modern *gyrB* sequences and the second ancient allele contain a G in that position. Most modern isolates have been identified with a methionine in position 543 of *gyrB* in reference strain AB014215 whereas the ancient Kwäday Dän Ts'ìnchi strain and the modern Ky679 Japanese isolate contain an isoleucine (Figure 4.8).

CACATCAACCGCACCAAGAACGCGATTCATAGCAGCATCGTGGACTTTTCCGG
CAAGGGCACCGGGCACGAGGTGGAGATCGCGAT**C/G**CAATGGAACGCCGGGTA
TTCGGAGTCGGTGCACACCTTCGCCAACACCATCAACACCCACGAGGGCGGCA
CCCACGAAGAGGGCTTCCGCAGCGCGCTGACGTCGGTGGTGAACAAGTACGCC
AAGGACCGCAAGCTACTGAAGGACAAGGACCCCAACCTCACCGGTGACGATAT
CCGGGAAGGCCTGGCCGCTGTGATCTCGGTGAAGGTCAGCGAACCGCAGTTCG
AGGGCCAGACCAAGACCAAGTTGGGCAACACCGAGGTCAAATCGTTTGTGCAG
AAGGTCTGTAACGAACAGCTGACCCACTGGTTTGAAGCCAACCCCACCGACGC
GAAAGTCGTTGTGAACAAGGCTGTGTCCTCGGCGCAAGCCCGTATCGCGGCAC
GTAAGGCACGAGAGTTGGTG

Figure 4.7. The 495 bp sequence of the Kwäday Dän Ts'ìnchi *M. tuberculosis gyrB* gene indicating the presence of two nucleotides in one base position.

HINRTKNAIHSSIVDFSGKGTGHEVEIA**I**QWNAGYSESVHTFANTINTHEGGTHEEGFRS
ALTSVVNKYAKDRKLLKDKDPNLTGDDIREGLAAVISVKVSEPQFEGQTKTKLGNTEVKS
FVQKVCNEQLTHWFEANPTDAKVVVNKAVSSAQARIAARKARELV

Figure 4.8. The Kwäday Dän Ts'ìnchi *M. tuberculosis gyrB* amino acid sequence

4.3.3.2 *katG*

163 bp of the *katG* gene from the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain were amplified and sequenced (Figure 4.9). The sequence was identified to be 100 % identical with numerous modern *M. tuberculosis* strains. The identification of guanine in position *katG* $^{1388\ G}$ (KatG463 cgg) is interesting because this nucleotide has been identified in lineage II modern strains that are associated with European strains (11) (Figure 4.10).

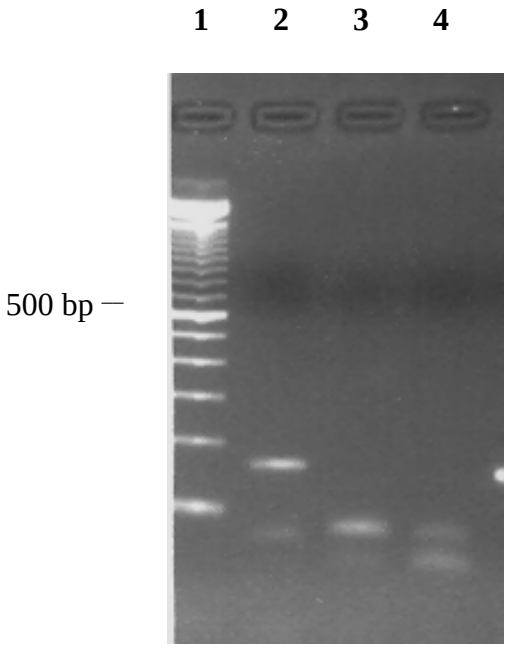

Figure 4.9. Amplified PCR products (163 bp) using primers katGF and katGR (78) on a 2% agarose gel. **Lane 1**, 100 bp ladder (New England Biolabs Inc, Pickering, ON, CAN.); **Lane 2**, stomach extraction; **Lane 3**, negative extraction; **Lane 4**, negative PCR.

TCAGCCACGACCTCGTCGGCGAAGCCGAGATTGCCAGCCTTAAGAGCCAGATC
C**G**GGCATCGGGATTGACTGTCTCACAGCTAGTTTCGACCGCATGGGCGGCGGC
GTCGTCGTTCCGTGGTAGCGACAAGCGCGGCGGCGCCAACGGTGGTCGCATCC
GCCT

Figure 4.10. Partial *katG* sequence (163 bp) associated with the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain indicating *katG* $^{1388\ G}$

4.4 DISCUSSION

4.4.1 The significance of identifying an ancient latent TB infection

The presence of *Mycobacterium tuberculosis* DNA in the lung tissue and possibly elsewhere without evidence of illness based on a complete histological exam, leads to the conclusion that the Kwäday Dän Ts'ìnchi ancient individual had a potential latent tuberculosis infection. The individual would have had no symptoms, and he would not have been infectious to his community. Latent tuberculosis infections are not well studied, and the location of the bacteria is not always apparent (5). Lung granulomas, lymph nodes, or tissues farther away from the initial site of infection have all been suggested (48). It is also possible that viable bacteria may reside outside granulomas, in areas unaffected by the infection (49).

Superficially normal lung tissues were analyzed by Hernandez-Pando and colleagues from 47 Mexican and Ethiopian individuals whose causes of death were unrelated to tuberculosis (50). They used PCR to identify the presence of *M. tuberculosis* DNA which resulted in the identification of latent tuberculosis infections in 15 of the individuals. This indicated that the presence of a granuloma is not necessary for evidence

of an *M. tuberculosis* infection. Interestingly, the researchers noted that while a tuberculosis infection was not evident, a histological examination revealed that all of the positive tissues contained accumulations of carbon particles in the macrophages or nearby fibrous tissue and the subpleural space. Carbonaceous material was identified in the lungs of Kwäday Dän Ts'ìnchi (Straathof, unpublished). It is not unusual though to find evidence of smoke inhalation in the lungs of an individual from a pre-modern era.

It is assumed in paleomicrobiology studies that when ancient *M. tuberculosis* DNA is identified in pathologic tissues other than the lungs, the individual suffered from the symptoms of the disease. There is still uncertainty of the significance of a positive identification of ancient *M. tuberculosis* DNA in non-remarkable bone or tissue. The amplification of *M. tuberculosis* DNA from multiple areas of the individual was previously thought to be the result of a systemic spread of the bacteria (51). Multiple Kwäday Dän Ts'ìnchi tissue samples tested positive for *M. tuberculosis* complex DNA. Only the lung sample was retested at the Hebrew University in Jerusalem and the positive identification was confirmed. Unfortunately, confirmation from another laboratory of multiple organ involvement was not possible due to the limited amount of remaining tissue.

4.4.2 The identification of the *M. tuberculosis* complex and specifically *M. tuberculosis*

Primers designed by Eisenbach and colleagues (43) were used on multiple Kwäday Dän Ts'ìnchi tissues to amplify 123 bp of IS*6100*. This region was identified as part of an insertion sequence that was specific for the mycobacterial species associated with the *M. tuberculosis* complex (52). Multiple copies of the IS*6100* sequence have been identified in most of the members of the *M. tuberculosis* complex, whereas *M. bovis* generally contains

only one copy (53). Since six mycobacterial species form the *M. tuberculosis* complex, further genetic analyses were performed to confirm a *M. tuberculosis* specific infection.

The amplification of an IS*6100* sequence fragment to identify an *M. tuberculosis* complex infection has been reported in numerous ancient tuberculosis studies (18, 25, 54, 55, 56, 57). In a recent study by Coros and colleagues, a similar insertion sequence was identified in *M. smegmatis* (58). While the sequence was not identical to the IS*6100* associated with the *M. tuberculosis* complex, it was similar enough to indicate a connection between IS*6100* and other mycobacteria. Based on this finding, it is critical to diagnose ancient *M. tuberculosis* infections on more than a positive amplification of a portion of the IS*6100* insertion sequence. In the following sections, different genomic regions that were analyzed to specifically characterize the Kwäday Dän Ts'ìnchi *M. tuberculosis* complex infection will be outlined.

4.4.2.1 *Rv3479*

The *Rv3479* gene, which is 3063 bp long, encodes a possible transmembrane protein in *M. tuberculosis* strain H37Rv. *M. bovis* AF2122/97 has a 714 bp deletion in *Rv3479* that results in two smaller open reading frames (59). Primers were used on Kwäday Dän Ts'ìnchi tissue extract to amplify a 156 bp region within *Rv3479* that is not present in *M. bovis* thus confirming that the bacterium was not *M. bovis*. These results indicate that deleted regions are useful for ruling out members of the *M. tuberculosis* complex when characterizing a mycobacterial strain, especially if the region is found intact within *M. tuberculosis*.

4.4.2.2 TbD1

Previously published primers (34) were chosen to test the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain for an *M. tuberculosis* specific TbD1 deletion. Modern *M. tuberculosis* isolates can be separated into genetically modern or ancestral strains based on the presence or absence of a deletion (TbD1) (45). When present, this 2153 bp fragment consists of the *mmpS6* and *mmpL6* genes, whereas in TbD1 strains, these genes are absent and truncated respectively. Strains with the TbD1 region present usually have few or no copies of IS*6100* and are considered 'ancestral' strains to the 'modern' strains lacking the TbD1 region (45). Studies have shown that the ancestral *M. tuberculosis* strains have a CTG codon at $katG^{463}$ which indicates that the CGG point mutation found in many strains including the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain occurred after the deletion of TbD1 (45).

Because of limited genetic variation between *M. tuberculosis* strains, researchers have suggested that the modern strain encountered a bottleneck at some point in history where only a small number of the bacterial population reproduced, and they have recommended the testing of mummified individuals for the presence or absence of the TbD1 region in those individuals positive for a *M. tuberculosis* infection (45). A recent study on the use of the spoligotyping method to characterize the *M. tuberculosis* complex, strains that were associated with three ancient individuals from Thebes West, Upper Egypt revealed a spacer pattern that is similar to spoligotype patterns from modern *M. tuberculosis* isolates that have the TbD1 deletion. Based on this evidence, the investigators identified that 'modern' *M. tuberculosis* strains were likely present by 500 BC in that region (60).

Hershkovitz and colleagues studied osteological samples approximately 9000 years old from the remains of a child and a female recovered in Atlit-Yam, an early village with evidence of domestication (57). They identified the presence of *M. tuberculosis* DNA in the remains, and through deletion analysis, they determined that the TbD1 deletion existed 9000 years ago. The identification of the TbD1 deleted region in the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain indicates that 'modern' strains were also present in North America approximately 200 to 300 years ago.

4.4.3 Additional genetic analyses

In addition to the *Rv3479* and TbD1 analyses, fragments of the *katG* and *gyrB* genes were amplified. This was necessary for further characterization of the ancient *M. tuberculosis* strain. Unfortunately, not enough sequence information was obtained for a phylogenetic analysis with modern strains, but an interesting polymorphism was determined in the *katG* sequence, and two *gyrB* alleles were identified that suggest the presence of a selective pressure.

4.4.3.1 *gyrB*

Along with other pathogens, the genome of *M. tuberculosis* contains genes *gyrA* and *gyrB* that encode two A subunits and two B subunits of a DNA gyrase, which is a type II topoisomerase that is involved with DNA unwinding for the purpose of replication (61). Modern drug therapies such as fluoroquinolones are used to target this locus to inhibit transcription (62).

A portion of the Kwäday Dän Ts'ìnchi *M. tuberculosis gyrB* gene was amplified and sequenced, because recent studies have shown that *gyrB* sequence polymorphism analysis is another approach to resolve the species type within the *M. tuberculosis* complex (63, 64). The ancient *gyrB* sequence is very similar to modern *M. tuberculosis gyrB* sequences, which confirms the finding that the strain is indeed *M. tuberculosis*. Interestingly though, two *gyrB* alleles were identified to be associated with the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain. Mokrousov and colleagues studied this region in modern strains from northwestern Russia and discovered a high number of isolates that contained both the mutant and wild-type alleles of *gyrA* or *gyrB* (61).

Kasai and colleagues analyzed *gyrB* sequences to determine the role of *gyrB* for identification within the *M. tuberculosis* complex (47). They found polymorphisms that were unique to members of the complex. The substitutions were synonymous, and the authors note that this was likely due to natural occurring events and not from drug therapy because antibiotic resistance is the result of nonsynonymous substitutions.

Interestingly, the 495 bp *gyrB* sequence amplified from the tissue of Kwäday Dän Ts'ìnchi shares a 100% identity with strain KY673 from the Kasai et al. study. A unique polymorphism was identified in KY673 that was also identified in the Kwäday Dän Ts'ìnchi *M. tuberculosis gyrB* sequence. A G nucleotide normally located in position 1117 is a C in both KY673 and one 'allele' of the Kwäday Dän Ts'ìnchi strain. This base substitution is nonsynonymous and results in an amino acid change from methionine to isoleucine. This leads to the question of how the ancient strain acquired a polymorphism that resulted in an amino acid change prior to the antibiotic era.

4.4.3.1.1 *Salicornia:* A precontact antibiotic?

Anti-gyrase activity has been found in natural compounds. The identification of CcdB and microcin B17 proteins with topoisomerase activity has resulted in the possibility of new antibiotic development (65). The knowledge that anti-gyrase activity can exist in natural compounds suggested a closer look at the diet reconstruction data. Mudie and colleagues analyzed the pollen grains recovered from the stomach of the Kwäday Dän Ts'ìnchi ancient individual and discovered an abundance of Chenopodiaceae pollen (66). Through the use of environmental scanning electron microscopy, they confirmed a large percentage of the pollen found in a stomach sample was *Salicornia perennis* (glasswort) which has been used as food and medicine. This was an unexpected finding for the investigators because plants from the Chenopodiaceae family are not common in the region (67).

Was it possible that *Salicornia* may have been consumed for medicinal purposes? A literature search for publications that connected *Salicornia* with the disease tuberculosis was unsuccessful, but a Google search produced a site that described the granting of a patent to a group of scientists for their discovery of the antimycobacterial properties of *Salicornia brachiata* (68). Unfortunately, a screening of British Columbian medicinal plants by McCutcheon and colleagues for antimycobacterial properties did not include *Salicornia* (69). Even if the Kwäday Dän Ts'ìnchi ancient individual had not consumed *Salicornia* for medicinal purposes, the possibility remains that the ingestion of this natural compound may have provided the selective pressure required to generate a non-synonymous amino acid substitution.

4.4.3.2 *katG*

A 163 bp fragment of the *katG* gene from the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain was amplified and sequenced, and a guanine base was identified in position *katG* $^{1388\ G}$. The sequence was 100% identical to numerous modern strains, which was expected considering the low genetic variability of the *M. tuberculosis* genome. Since the sequence was 100% identical, the *katG* $^{1388\ G}$ is likely not a result of misincorporation due to DNA damage.

KatG has been identified with catalase and peroxidase activity and plays an important role in the function of the bacterium (70). Gagneux and colleagues suggested that there is a link between the various mycobacterial lineages and human populations, which may have implications for the control of tuberculosis (70). They demonstrated that *M. tuberculosis* is defined by six phylogeographical lineages and the *katG* codon at position 463 was used as one of the markers to characterize the lineages. The Euro-American lineage was defined as consisting of a *katG* 463 CGG codon. They noted also that sub lineages of the Euro-American lineage were found in Africa and the Middle East.

Modern *M. tuberculosis* isolates have been classified into three genotypic groups based on single nucleotide polymorphisms found in *katG*. Group 1 has the codon CTG (Leu) at *katG* 463, and Groups 2 and 3 are defined as having the codon CGG (Arg) (71). Group 1 is considered to be evolutionary the oldest (46). The Kwäday Dän Ts'ìnchi *M. tuberculosis* strain belongs to Group 2 or 3 based on the finding of the CGG codon.

A recent study of silent nucleotide polymorphisms in *Mycobacterium tuberculosis* by Baker and colleagues defined four main lineages of modern strains that significantly corresponded with geographical regions (11). Group 1 isolates could be divided into

lineage I, III and IV whereas group 2 and 3 isolates comprised lineage II. Lineage IV was found globally, whereas lineage I and III were associated with southeastern Asia and India respectively. Lineage II was significantly associated with Europe.

Zink and colleagues included an analysis of *katG* in their study of strain identification of *M. tuberculosis* complex DNA in archival tissue samples (34). They determined that the ancient *M. tuberculosis* strains also corresponded with the modern genetic groups 2 and 3, which is not surprising considering that the samples originated in Europe. Since the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain was typed also as group 2 or 3, this suggests that it would be similar to the lineage II isolates from the Baker et al. study (11) that were significantly associated with the European geographical region. This evidence advances the idea that the Kwäday Dän Ts'ìnchi ancient individual lived in a post-European contact time, as was suggested by the radiocarbon dates, but the molecular data can not confirm that the Europeans had a constant presence in the region.

4.4.4 Relevance of a protohistoric *M. tuberculosis* infection on the Northwest Coast

Previous studies of pre-contact skeletal samples (representing 193 individuals) from the northern coast of Alaska and the eastern Aleutian Islands identified no specific osteological indicators of tuberculosis (72). This research suggests that either *M. tuberculosis* was not present in the region or pre-contact infected individuals died without any skeletal impact. Historical documents indicate that the first contact between Europeans and the local inhabitants of the Northwest Coast was 1778 (36). The identification of a protohistoric latent *M. tuberculosis* infection with a genetic connection to European strains reinforces the impact of European colonization.

Scientists originally thought that all *M. tuberculosis* strains had the same capacity of virulence but strain genotyping based on human population groups indicates that some strains are more infectious and spread easier (9). The Beijing family of strains are currently recognized as the most widespread and have caused outbreaks in many locations in Asia and North America (9). Researchers have suggested that while skeletal and molecular evidence indicates that tuberculosis has affected humans for thousands of years, the modern TB epidemic began in Europe in the 1700s before spreading to the rest of the world (73). Based on the limited analysis of the Kwäday Dän Ts'ìnchi *M. tuberculosis* strain, one can only speculate that this strain is associated with the modern epidemic.

4.4.5 Modern Impact of Tuberculosis on Northwest Coast populations

Historical documents from post-European contact to the present indicate high infection rates of tuberculosis among Canadian Aboriginal peoples (74). Medical records from Alaska, USA also indicate a higher infection rate among the Aboriginal Alaskans. Gaenslen and colleagues analyzed the records from the Mt. Edgecumbe Alaska Native Health Service Hospital for a 10 year period between 1953 and 1963 (75). The investigators identified evidence of a high incidence of TB among Aboriginal Alaskans, and 11 patients were noted as having TB meningitis. Three of these individuals developed meningitis even after receiving treatment for tuberculosis.

In the 1990s, Canadian First Nations communities on reserves were found to have infection rates eight to ten times higher than the Canadian rate (76). While socioeconomic factors such as crowding have played a role in the transmission of the bacterium, genetic studies of the host-pathogen interactions associated with these populations needs to be

addressed. The modern populations are still dealing with the impact of this disease because the strategies for handling the infection have not been as successful as was expected. The genotyping of ancient *M. tuberculosis* strains from the area may result in a better awareness of strain virulence in First Nation communities.

4.5 CONCLUSION

Mycobacterium tuberculosis complex DNA was identified in the lung tissue of the Kwäday Dän Ts'ìnchi ancient individual. Through the analysis of the *Rv3479* gene and the TbD1 region, the infection was confirmed as specifically *M. tuberculosis*. Since there was no evidence of illness, this individual likely had a latent tuberculosis infection. The ancient TB strain was characterized through genetic analyses involving the *katG* and *gyrB* regions. The presence of a single nucleotide polymorphism (G) at position 1388 of the *katG* gene places the strain in Lineage II, which is more commonly associated with European *M. tuberculosis* strains. The identification of two *gyrB* alleles that differ by one amino acid suggests the presence of a selective pressure, but the individual lived in a time prior to the usage of modern antibiotics. Perhaps the identification of a possible latent *M. tuberculosis* infection and the presence of *Salicornia* pollen in the stomach with potential antitubercular properties may result in more research into the beneficial properties of traditional plants.

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CHAPTER FIVE: Discussion

The microbial DNA analysis of Kwäday Dän Ts'ìnchi's tissues involved specific PCR tests for the presence of genetic material from *Mycobacterium tuberculosis* and *Helicobacter pylori*. DNA from both bacteria was positively identified. The radiocarbon dating of artifact samples and collagen tissues indicated a timeframe of approximately AD 1670 and 1850 (Richards et al. 2007), which is either pre-European contact or early European contact; therefore I could not conclusively determine that these bacteria were present prior to the arrival of Europeans in the Canadian Northwest.

Interestingly, my characterization of the ancient microbial genetic material revealed evidence linking both bacteria to European strains. *H. pylori vacA* s2 and m2a alleles of the *vacA* signal region and middle region respectively were identified in the stomach tissue of the Kwäday Dän Ts'ìnchi individual. This indicates the possibility that an indigenous strain may have recombined with a European strain, since *vacA* s2 and m2a alleles are often associated with European strains (Yamazaki et al. 2005, Ghose et al. 2002).

A connection with Europe was also identified in the ancient *M. tuberculosis* strain identified in the lung tissue of the Kwäday Dän Ts'ìnchi individual. An analysis of polymorphisms in the *katG* gene indicated an association with Lineage II strains according to Baker et al (2004). In their study of neutral genetic variation in genes associated with drug resistance, the investigators identified a significant association between continent of

birth and lineage of the *M. tuberculosis* strain. Europe was identified as specifically associated with Lineage II.

H. pylori and *M. tuberculosis* are not morphologically or biologically similar bacteria yet they share an important feature that is relevant to the study of ancient human populations. Scientists have identified through molecular technology that *M. tuberculosis* has been associated with humans for thousands of years, and phylogenetic analysis of modern *H. pylori* strains have also revealed a lengthy association with humans. These pathogens have developed relationships with their human hosts that result in a glimpse of past human migration through the differences identified in the bacterial strains both temporally and spatially.

The decision to test the tissues specifically for these bacteria was based partly on the original radiocarbon analysis that suggested the individual was approximately 550 years old and pre-European contact (Beattie et al. 2000). I thought that it would be interesting to analyze microbial genetic data for evidence of illness in addition to migration patterns prior to the influence of European strains. A reappraisal of the original radiocarbon dates by another research team revealed that the individual was more recent in time (Richards et al. 2007). Unfortunately the new dates (AD 1670- 1850) leave a question as to whether the site was from the pre- or post-European contact period. With these new dates in mind, I anticipated that perhaps the microbial genetic data would clarify the time period of the site.

The characterization of both ancient *H. pylori* and *M. tuberculosis* strains associated with the individual suggest a European connection. It does not confirm a constant presence of Europeans in that particular area, but it does rule out the pre-contact time period with no European influence. The Kwäday Dän Ts'ìnchi ancient individual may have lived during

the protohistoric era at a time when the presence of Europeans was experienced through trade.

The other reason to test for both *M. tuberculosis* and *H. pylori* was based on the current world-wide infection rates of both bacteria. Reports suggest that a third of individuals are infected with *M. tuberculosis* and one half of all humans world-wide are infected with *H. pylori* (World Health Organization 2008, Atherton 2006). Approximately 10% of those infected with either bacterium have severe symptoms, with alarming infection rates in developing countries. Pathological and histological assessments of the tissues associated with the Kwäday Dän Ts'ìnchi ancient individual did not reveal any evidence of infection, but I felt it was worthwhile to test for microbial DNA evidence of infectious diseases that are highly probable and still currently world-wide issues.

Many potential benefits exist for the identification of microbial DNA in ancient material. In addition to confirming paleopathological diagnoses for epidemiological studies, the evolution of the bacteria and its association with the human host can be clarified. Lengthy temporal associations with humans suggest that perhaps some of these micro-organisms are benign or possibly even beneficial to the human hosts that they infect. Investigators have suggested that this may be the case with the bacterium *H. pylori*. Evidence has been identified that *H. pylori* colonization results in lower stomach acid levels which is protective against gastro-esophageal reflux (Blaser 1998).

It is likely that the ancient individual did not suffer from any gastric symptoms as a result of the *H. pylori* infection in his stomach. The signal region of the virulence-associated gene *vacA* was typed as s2, which is more commonly identified in infections that do not result in disease (Letley et al. 2003). The identification of *M. tuberculosis* DNA

without evidence of immune system involvement suggests that the individual had a potential latent tuberculosis infection, and he would not have had any symptoms of the disease tuberculosis.

5.1 Technical lessons learned

The field of paleomicrobiology continues to evolve towards the use of standardized methods to ensure the veracity of the work and to prevent or at least minimize issues such as contamination. Drancourt et al. (2005) and Roberts and Ingham (2008) reviewed paleomicrobiology publications to assess the quality of the data, and many of the studies were found to be lacking in their descriptions of how the issue of DNA contamination was considered. Following, I will expand on my methodology that I described in chapters three and four, with reference to the criteria for ancient microbial DNA research that was recently summarized in the Roberts and Ingham (2008) review paper:

- **Sterile sampling at the initial excavation**

The remains of the Kwäday Dän Ts'ìnchi ancient individual were handled with care and respect by all researchers, and a well-thought out plan was in place by the Management Committee to limit contamination of the tissues. Beattie et al. (2000) detailed the process upon which the remains were excavated. The excavators wore Tyvek® suits and sterile latex gloves. The remains were wrapped in two layers of sterile fabric hospital wraps, covered with sterile plastic sheeting and placed in clean plastic containers. The containers were kept in the snow to maintain a cold temperature until they were flown to Whitehorse and locked in a chest freezer at -17˚C. Once an agreement was reached between the

Champagne and Aishihik First Nations and the British Columbia Archaeology Branch, the remains were flown to Victoria and placed in a locked walk-in freezer at the Royal British Columbia Museum (Beattie et al. 2000). The samples for the microbial DNA assessment were not retrieved until the third autopsy, which I attended. Unfortunately, since it is normal procedure during an autopsy to use the same instruments between organs, I could not rule out the contamination of bacterial DNA from one organ to another.

- **The use of physically isolated and dedicated work areas**

DNA extraction of the Kwäday Dän Ts'ìnchi tissue samples and PCR set-up was undertaken in a contained lab where no other molecular research was being performed, and no amplified genetic material was present. The thermal cycler and equipment for the post-PCR process was located in a different laboratory.

- **The development of a DNA history for lab personnel and past projects**

During the multiple autopsies of the ancient individual and during DNA extraction, face masks were worn for protection of the researchers as well as to protect the tissue samples from possible contamination. It is not believed that any team member had an active case of tuberculosis that would result in tissue contamination. It is also known that *H. pylori* is not easily transmitted, and a direct source of the bacteria such as bacteria-laden vomit is required for transmission (Amieva and El-Omar 2008). I was the only individual at the University of Saskatchewan to work with the tissue samples post autopsy, which also limited the number of contamination sources.

- **The use of protective clothing**

Researchers have discovered that if *M. tuberculosis* is viable and exposed to the environment during autopsy, the team members risk contracting the infection. Molecular methods were used to confirm the transmission of *M. tuberculosis* to an embalmer from a cadaver (Sterling et al. 2000). Studies have also been undertaken on preserved tissues. Kappel et al. (1996) reviewed all literature regarding the impact of formalin on *M. tuberculosis* to determine the disinfection efficacy. The investigators could not conclusively rule out the risks, and they determined that more studies were needed to establish guidelines to ensure the safety of those individuals who work with formalin-fixed *M. tuberculosis* infected tissue. It was important to use protective clothing during both the Kwäday Dän Ts'ìnchi autopsy and all molecular analyses for the safety of the researchers and to prevent contamination of the samples.

- **The removal of surface contamination from the samples**

The Kwäday Dän Ts'ìnchi tissue samples for the microbial DNA analysis were very small with most weighing less than 0.2 grams, and I was unable to effectively remove any surface tissue prior to DNA extraction on most of the samples. In future studies, I would recommend adhering to the suggestion of surface removal, because if I had enough tissue to remove potentially contaminated surfaces, a study of the presence of *M. tuberculosis* bacteria in multiple tissues from an ancient individual with a latent tuberculosis infection may have been possible.

The surface of the rib sample was the only sample surface that was treated differently. I soaked the rib for 15 minutes in six percent sodium hypochlorite (full-

strength bleach) and rinsed it multiple times with sterile water. Bleach is commonly used to remove surface contamination from bones and teeth (Kemp and Smith 2005). Kemp and Smith (2005) identified that a bone should be immersed in a minimum of three percent sodium hypochlorite for at least 15 minutes. They also discovered that the endogenous DNA was stable in a six percent sodium hypochlorite treatment for 21 hours.

- **Evidence of amplifiable DNA**

When mitochondrial and nuclear DNA are identified in an ancient sample, this suggests that microbial DNA, if present, may also be intact since it was affected by the same taphonomic processes as the other DNA. A separate research team identified the presence of the ancient individual's mitochondrial DNA. Monsalve et al. (2002) extracted the mitochondrial DNA (mtDNA) and determined that the DNA belonged to haplogroup A. Aboriginal Americans are known to belong to one of five haplogroups; A, B, C, D and X (Torroni et al. 1993, Brown et al. 1998). Haplogroup A is the most common North American type and has been identified in Aboriginal individuals from the Northwest Coast (Monsalve et al. 2002). A tissue study revealed remarkable preservation in those tissues that remained frozen, such as the lung tissue (Monsalve et al. 2008). The investigators found little evidence of postmortem decay in the frozen tissues and suggested that the tissues froze soon after the death of the individual. I was confident that since the ancient individual was preserved within glacial ice and the tissues were only thawed during the autopsies, it seemed reasonable that amplification of microbial DNA would be possible, especially with the knowledge that mitochondrial DNA was successfully amplified.

- **The use of negative controls during both DNA extraction and amplification**

Negative controls were used in this microbial DNA study but as I will expand on further in a following section, it is wise to set up more than one negative extraction control.

- **Appropriate molecular behaviour**

Due to the likelihood that the ancient DNA is damaged, there should be an inverse relationship between the successful amplification and the size of the DNA. Long DNA sequences are not expected and if a long stretch is amplifiable, modern DNA contamination should be considered. The successful Kwäday Dän Ts'ìnchi microbial DNA amplifications were mainly between the lengths of 100 basepairs and 300 basepairs.

- **The ancient sequence should make phylogenetic sense.**

A group of related organisms share an evolutionary history that can be identified through DNA sequence analysis and represented visually as a 'tree.' In this regard, I had interesting results with respect to the *H. pylori* DNA amplification from the stomach tissue of Kwäday Dän Ts'ìnchi. Even though ancient DNA sequences are mainly between 100 and 300 basepairs long, the short sequences from the *H. pylori vacA* gene were specific enough to be placed within a grouping of sequences from other modern Alaskan strains in a phylogenetic tree. It was fascinating to discover that a bacterium associated with an individual recovered from the Northwest Coast shared sequence similarities with modern isolates close to the same geographical location where the ancient individual was recovered. Unfortunately, the different strains of *M. tuberculosis* are very genetically similar, and the phylogenetic data only indicated that the ancient strain was identical to

modern strains in the regions that were compared. This was not surprising considering that the bacterial DNA was identified using known conserved genetic regions.

- **Reproducible results and independent verification if the results were unexpected.**

While I was unable to have another laboratory confirm the *H. pylori* data due to limited stomach tissue, the *M. tuberculosis* identification in the lung sample was confirmed by Dr. M. Spigelman of the Hebrew University. This was fortunate because although I could rely on the phylogenetic analysis as a measure of authenticity of the *H. pylori* amplifications, this was not possible for the *M. tuberculosis* sequences.

5.1.1 Sample size

If I had an opportunity to revisit the studies on the identification of *M. tuberculosis* and *H. pylori* in Kwäday Dän Ts'ìnchi tissues, I would first request additional samples. The KDT Management team was most gracious in providing numerous tissue samples for the microbial DNA study, and it was my own decision to ask for less than half a gram per sample. At the time of sample retrieval, with knowing that PCR technology can be very sensitive and only a small amount of DNA extract is required, I thought it prudent to request the smallest amount possible as a way of showing respect for the decision to allow the testing. Needless to say, I learned that although the amount of tissue I had was reasonable for a primary screening, I did not have enough for repeated experiments at the University of Saskatchewan or by other researchers for confirmation of the results (Table 5.1).

Table 5.1 Summary of Kwäday Dän Ts'ìnchi tissue results

Kwäday Dän Ts'ìnchi tissue samples	Catalogue number	Weight (g)	Number of extractions	Primer pairs that resulted in successful PCR amplifications	Number of Sequences (forward and reverse)
Descending colon	T-4-8	0.15	1	P1/P2	1
Spleen (possible)	T-23	0.04	1	P1/P2	1
Stomach contents	T-27-1	0.49	1		
Liver	T-33-1	0.18	1	P1/P2 MTB1/MTB2	1 1
Lung	T-34-1	0.46	2[a]	P1/P2[b] MTB1/MTB2	1 (reamplification of PCR product from 1st extraction) 1
Myocardium	T-37-1	0.08	1	P1/P2	1
Small intestine	T-38-4	0.03	1	P1/P2	1
Stomach	T-38-5	0.24	2[c]	MF1/MR1 – 2 VA1X/VA1XR – 2 (very low conc., samples were combined for sequencing) Y98vacAmF/R – 2 flaA-1-F/2-R - 2 flaA-3-F/3-R – 2 P1/P2 TSgyrBF/R, TSgyrB2F/R, TSgyrB3F/R, KatGF/R[d]	1+1 (reamplification of PCR product) 1+1 (reamplification of PCR product) 2 2 2 1 1 1
Cecum	T-38-8	0.03	1	P1/P2	1
Lymph node (mediastinal)	T-39-1	0.22	1	P1/P2	1
Rib (right 4th)	T-40	0.95	2		
Skin	T-41	0.01	1		
Colon contents	T-44	0.26	1		

[a] 1st extraction – positive P1/P2 on gel but sequencing was not successful due to low concentration of PCR product, 2nd extraction – the supernatant was split into 3 samples before using the silica columns

[b] independently replicated by Dr. M. Spigelman of the Hebrew University

[c] stomach tissue was extracted using a QIAGEN QIAamp® DNA Mini Kit, and all other tissues were extracted using a QIAquick® PCR Purification Kit (no stomach tissue was remaining to compare methods)

[d] due to a higher volume of stomach extract, and since it indicated a positive P1/P2 amplification, this extract was used for the additional *M. tuberculosis* testing

While it is usually not necessary to always have PCR results validated, in circumstances where the finding is unusual, it is important to have independent verification. Fortunately, the lung sample that I received was much larger than the other samples. I was authorized by the KDT committee to send Dr. Victoria Monsalve at UBC a lung sample (0.65 grams) for a study on tissue preservation (see Monsalve et al. 2008). She shared part of the lung tissue with Dr. Mark Spigelman, a published ancient tuberculosis researcher, for the confirmation of *M. tuberculosis* complex DNA in the tissue sample.

5.1.2 Negative controls

It is common knowledge that negative controls during DNA extraction and amplification are beneficial for spotting possible sources of contamination. In future studies, I will use more than one negative tissue extraction control and avoid extracting different tissues at the same time. Although I amplified *M. tuberculosis* DNA in multiple Kwäday Dän Ts'ìnchi tissues, including a mediastinal lymph node and abdominal organ tissue, the one extraction control was sporadically showing DNA bands upon amplification of the multiple-copy insertion sequence IS*6100*. This indicated the possibility that some of the positive tissues were contaminated with *M. tuberculosis* DNA. The finding of *M. tuberculosis* DNA in the lung tissue resulted in the identification of a latent tuberculosis infection. It would have been interesting to have evidence of a hematogenous spread because the bacterial locations in latent infections are still unclear. Interestingly, investigators have discovered that some *M. tuberculosis* genotypes are associated with the involvement of different organs (Thwaites et al. 2008).

Future ancient microbial studies where multiple organ involvement might be identified should include measures for controlling contamination between the tissue samples from the same individual. This is straight forward once the samples are in the lab, but although sterile instruments are initially used at the autopsy, it is important to recognize the importance of replacing the instruments before each organ inspection.

CHAPTER SIX: Conclusions

This thesis documents the study of ancient microbial DNA in tissues from the Kwäday Dän Ts'ìnchi ancient individual who was recovered from a melting glacier in northwest British Columbia. When this microbial DNA project was first proposed, the original intention was to identify any genetic evidence of normal bacterial flora or pathogenic bacteria through the use of polymerase chain reaction (PCR) with universal primers. This methodology resulted in positive amplifications from the negative PCR controls. Since I could not be confident that the genetic material from authentic ancient bacteria was amplified when universal primers were used, I chose to test the tissues for the microbial DNA from two specific bacteria: *Mycobacterium tuberculosis* and *Helicobacter pylori*. High modern infection rates and the interesting connection with ancient human migration made this decision reasonable.

Helicobacter pylori DNA was amplified and sequenced from the stomach tissue of the ancient individual. Through an analysis of the *H. pylori vacA* gene, a hybrid m2a/m1d allele and an s2 signal region allele were identified. I also amplified and sequenced a portion of the *flaA* gene. The amplification of a *cagA* gene fragment was unsuccessful, but I could not confirm that the strain was *cagA* negative due to the possibility of DNA damage in that region.

Modern *H. pylori* infection rates of Aboriginal individuals in the circumpolar regions are high, and some treatments have been unsuccessful due to antibiotic resistance.

The strain associated with the Kwäday Dän Ts'ìnchi ancient individual carried a *vacA* s2 allele, which is an indication that the strain was non-toxigenic. The presence of an s2 allele, which is unusual in Asian strains, suggests that European strains were present during the timeframe of AD 1670 to 1850. The characterization of the *vacA* m region revealed a hybrid region that is rare in modern strains. The phylogenetic analysis indicated that the m1d region clustered with previously studied novel Native American strains that were closely related to Asian strains. These observations are consistent with the theory that the first humans into the New World migrated over the Bering Strait from Asia.

I also identified *Mycobacterium tuberculosis* complex DNA in the lung tissue from the ancient individual. Through the analysis of the *Rv3479* gene and the TbD1 region, I confirmed the infection was specifically *M. tuberculosis*. Since there was no histological or pathological evidence of illness, I propose that this individual had a latent tuberculosis infection.

The ancient TB strain was characterized through genetic analyses involving the *katG* and *gyrB* regions. The presence of a single nucleotide polymorphism (G) at position 1388 of the *katG* gene places the strain in Lineage II according to Baker et al. (2004), which is more commonly associated with European *M. tuberculosis* strains. The identification of two *gyrB* alleles that differ by one amino acid suggests the presence of a selective pressure, but the individual lived in a time prior to the usage of antibiotics. The identification of a latent *M. tuberculosis* infection and the presence of *Salicornia* pollen with potential antitubercular properties in the stomach is an avenue for further research.

I would recommend testing for evidence of *H. pylori* and *M. tuberculosis* in future discoveries of mummified remains if the environmental conditions were optimal for DNA

survival, as was the situation with the Kwäday Dän Ts'ìnchi individual who had been preserved in glacial ice. Successful amplifications and analyses of the genetic material of these bacteria can lead to greater insights on the presence of two infectious diseases that have had long histories with humans but are still problematic in modern populations. This long association with humans also creates an interesting connection with the study of ancient human migration patterns through phylogenetic analyses of the relevant microbial DNA sequences.

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