MOLECULAR IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN THE MILWAUKEE COUNTY INSTITUTION GROUNDS CEMETERY

by

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A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

in Anthropology

at

The University of Wisconsin-Milwaukee

May 2015

ABSTRACT
MOLECULAR IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* IN THE MILWAUKEE COUNTY INSTITUTION GROUNDS CEMETERY

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The University of Wisconsin-Milwaukee, 2015
Under the Supervision of Professor Patricia Richards

The possibility of identifying *Mycobacterium tuberculosis* in skeletal remains has been a debated topic for many years. This study utilizes the remains from the 1991 and 1992 excavations of the Milwaukee County Institution Grounds Cemetery, a collection of human skeletons ranging from 1882 to 1925, of various ages and sexes, to address that possibility. To test the utility of previously used methods of osteological identification of tuberculosis, the collection has been analyzed for the IS6110 repetitive element marker using molecular biological techniques, such as Polymerase Chain Reaction (PCR). Eighty-six skeletons from the collection have been analyzed, with nine of them showing evidence of skeletal tuberculosis. PCR has also been carried out with the oxyR marker to rule out *Mycobacterium bovis* contamination on all positive IS6110 samples. The goal of the study was to evaluate whether or not osteological identification of *M. tuberculosis* is possible and whether it can be confirmed using molecular biological techniques.

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ACKNOWLEDGEMENTS

First and foremost, thank you to my advisor, Dr. Patricia Richards for her patience, support, advice and mentoring. I am continually grateful. Thank you also to my wonderful committee, Dr. Trudy Turner and Dr. Joseph P Gray for their advice and help. You have all made my thesis much stronger for your contributions.

Thank you to the Turner lab at the University of Wisconsin-Milwaukee for the generous use of your laboratory space and your thermocycler. Thank you to the Pepperell lab at the University of Wisconsin Madison for their generous contribution of tuberculosis DNA. Thank you to Dr. Kurt Reed for setting me up with the Pepperell lab and for giving me the idea for my thesis in the first place. Thank you to the DNA Sequencing Facility at the University of Wisconsin Madison and Jeremy Niece in particular for their help with my SBA gels and my results interpretation.

I am incredibly grateful to Carrie Jones and Emily Epstein for their help navigating the archaeology lab and the MCIG collection. In particular, Carrie's patience with my never ending questions and Emily's support during the nine months I needed it most. Thank you to all of my friends in the department who have joked, cried, studied, and commiserated with me.

I give a big thank you to my friends at home who have encouraged me to not spend all of my time thinking about school. Ellen, Molly, Erica and Sarah – you are all literally the best. Sarah in particular has shown me that you can still have babies and rock your career.

I would not have had the strength to go back to school and switch careers if it hasn't been for my family. My mom and sister for being my inspirations, my step-dad for his help and humor, and my dad for the numerous ways he has supported me and for giving me his interest in the macabre. The one person who I most literally could not have done this without is Zach. Thank you for being the only husband amazing enough to encourage me to spend hundreds on gas, take a low paying job, be away from home more and dedicate all of my free time to my career all because it makes me happy. You are quite literally amazing. All of my work is dedicated to William, who simply makes everything in my life better.

CHAPTER 1: INTRODUCTION

Tuberculosis is a disease with a long history. Until the mid-twentieth century and the popularization of antibiotics, tuberculosis claimed more lives than any other bacterial pathogen (Daniel 2006). This ancient disease, which has been found in the tissue of 5000 year old Egyptian mummies, is estimated to be approximately 150 million years old based on its rate of mutation (Daniels 2006). In the first decade of the twentieth century, tuberculosis accounted for 25% of the deaths of 5-44 year olds in the United States (Armstrong 1999). As late as the late 1900s tuberculosis was still claiming as many as five million lives per year worldwide with 62% of cases reported in Southeast Asia, 16% in sub-Saharan Africa, and approximately 8% in both of the Americas (Clark 1987, Cosivi 1998). Even with modern technologie, tuberculosis is still a threat to most of the world's population. Despite the invention of streptomycin in 1946 and the European vaccine campaign, this acid-fast bacillus snuck through the grasp of Western medicine and continues to claim lives in the developing world (Clark 1987, Daniels 2006). The World Health Organization estimated that 1.5 million people, mainly in Africa and Asia, died of tuberculosis in 2005 (Stone 2009).

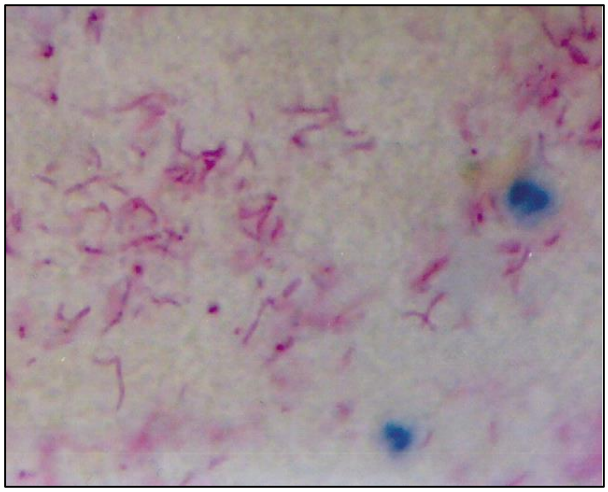

Figure 1.1 Ziehl-Neelsen stain of *Mycobacterium tuberculosis* from chest material (Donoghue 2004)

Tuberculosis disease in humans is most commonly caused by the bacterium *Mycobacterium tuberculosis* (Mtb), which was discovered by Robert Koch in 1882. Mycobacteria are acid-fast bacilli that are highly virulent and can manifest disease throughout the body, though infection in the pulmonary tract is most common (Smith 2003). The pulmonary form of tuberculosis causes "coughing, difficulty in breathing, weakness, lethargy, loss of appetite and weight, night sweats, pallor, and chest pain; if not treated the infection may spread to other organs including the skeleton, via the lymphatic and blood stream systems" (Stone 2009).

Tuberculosis is closely related to three other Mycobacteria: *M. bovis*, *M. microti*, and *M. africanum*. This group is referred to as the Mycobacterium tuberculosis Complex (MTC) and are so closely related that the symptoms of disease in humans can be difficult to distinguish clinically (Clark 1987, O'Brien 2004). The organisms that comprise the MTC show a greater than 99.9% similarity in DNA sequence (Smith 2003). Humans may become infected by either *M. tuberculosis* or by contact with animal populations infected with *M. bovis* through inhalation, ingestion, wound inoculation and rarely by congenital transmission (Burke 2011, O'Brien 2004). It is estimated that, at the start of the twentieth century, a significant number of individuals who

had tuberculosis disease were infected with *M. bovis*, due to its prevalence at that time, the clinically similar presentation of *M. bovis* to *M. tuberculosis*, and the lack of modern molecular biological detection techniques that could diagnose it as *M. tuberculosis* (Hardie 1992).

Tuberculosis is most commonly seen as a pulmonary disease, but may also infect bone, brain, and kidney tissue. The term "Latent Tuberculosis" refers to the condition of a person who has the tuberculosis microbe but is not in active stages of the disease, meaning that the characteristic pulmonary symptoms are not present. It is possible to have the *M. tuberculosis* and never suffer from the active disease, though this was unlikely before the rise of antibiotics (Burke 2011, Cockburn 1971). Latent tuberculosis is more common in individuals with strong immune systems who are occasionally able to clear the infection entirely. If an individual with latent Mtb experiences certain hardship and suffers from a weakening of his/her immune system, the disease can progress to the active stage (Burke 2011). Consequently, you see less latent tuberculosis in areas where there is overcrowding, poor hygiene, and other social stressors that lead to weakened immune systems (Smith 2003).

The infection of bone tissue by tuberculosis is colloquially referred to as Pott's disease. Pott's disease is depicted in early Egyptian art, defined by its characteristic bowing of the spine (Daniels 2006). In adult skeletons, these tuberculosis lesions are typically found in the lower back, the sacro-iliac joints, and the internal aspects of the ribs (Stone 2009). Once introduced into the circulatory system, tuberculosis will congregate in the hemopoietic tissues. The equilibrium of the "mesenchymal bone matrix-forming osteoblast cell lineage and the myeloid bone-resorbing osteoclast cell lineage" that is maintained in healthy bone is thereby breached (Meghji 1997). This causes the loss of the extracellular matrix which leads to the collapse of the spinal column.

It is unknown if a particular strain of Mtb is more likely to cause skeletal tuberculosis than others, but it has been shown previously that the most commonly virulent strain, H37Rv, contains the obligate protein cpn10 which has been shown to aid the bacterium in its infection of osseous tissues. Cpn10 is an osteolytic protein produced by Mtb which inhibits the proliferation of osteoblasts. Mycobacteria replicate within the skeletal lesions, releasing cpn10 so that the bone cannot repair itself through the regrowth of normal healthy osteoblasts (Roberts 2008, Meghji 1997).

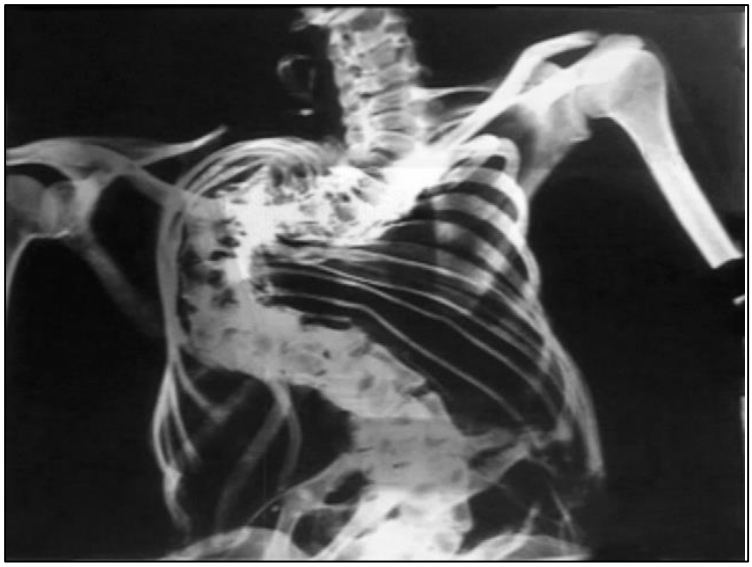

Figure 1.2 Spinal pathology from an eighteenth century Hungarian with Pott's disease (Donoghue 2004)

While it is not disputed that tuberculosis can infect bone tissue and cause Pott's disease, what has been an area of controversy is whether it is possible to correctly identify these signs in skeletal remains. Due to the similarity of skeletal tuberculosis to a host of other diseases, it has been speculated that it is not possible to say with confidence if an individual was infected by *Mycobacterium tuberculosis* based solely on his or her skeletal remains. For years, however, researchers have attempted to infer tuberculosis infection from remains found where there was a known prevalence of Mtb infection in the population. Looking for the most characteristic sign of

Pott's disease, the collapse of the vertebral bodies, as well as the beginning stages of osseous tuberculosis, many researchers have made assumptions about the infection status of their population of study. The question my research will strive to answer is this: Can you correctly identify tuberculosis in a skeletal population using osteological techniques, and can you confirm it using molecular biology?

CHAPTER TWO: REVIEW OF LITERATURE

There are a host of problems associated with attempting to infer tuberculosis infection using only skeletal remains. Due to the nature of how tuberculosis affects skeletal tissue, it can present similarly to a number of other diseases. There are also issues with attempting to determine anything about the population prevalence of tuberculosis from skeletal remains, as tuberculosis typically infects the osseous tissues of only 2-4% of its victims at the most, and less than 1% at the least (Raff 2006, Wilbur 2008). There is a subset of anthropologists, however, that believe that with the correct blend of technique and historical data, it is possible to determine whether an individual suffered from tuberculosis in his/her lifetime, and the prevalence of population infection as a whole (Roberts 2008, Roberts and Buikstra 2012).

The focus of paleomicrobiology during its relatively recent history has been the identification of microbial lesions in non-living bone. At the forefront of this field, Buikstra, Roberts, Stone, and Wilbur have published prolifically on the successful identification of tuberculosis in human skeletal remains. Stone and Wilbur argued in their 2012 contribution to Buikstra and Roberts' paleopathological anthology that the destruction of bone for molecular biological studies is not warranted when tuberculosis can be successfully identified from osteological evidence alone (Roberts 2008, Roberts and Buikstra 2012, Wilbur 2009). Wilbur *et al* stress that in order for a purely osteological analysis of tuberculosis to be successful the analysis must be rigorous and thorough (Wilbur 2009).

Figure 2.1 Typical spinal tuberculosis morphology (Nerlich 2009)

One of the hallmark indicators of tuberculosis in skeletal remains is the "destruction of the lower thoracic and/or lumbar vertebral bodies leading to spinal collapse and kyphosis" (Roberts 2009). This "very typical ventral collapse of the vertebral body leading to a more or less severe angulation of the vertebral column" (Figure 2.1) is more commonly seen as the end stage of osseous tuberculosis (Nerlich 2009). As discussed previously, this vertebral collapse is caused by the cpn10 protein's inhibition of new osteoblast proliferation which thereby inhibits the damaged bone tissue from rebuilding itself (Meghji 1997). The problem is that this complete vertebral collapse is far rarer than finding other more subtle indicators, those that are easily confused with other diseases. Pathognomonic lesions, or those that would be a specific indicator of tuberculosis, are non-existent. Rather, it is said that certain skeletal lesions are consistent with tuberculosis (Wilbur 2009). When the anterior aspect of the vertebral bodies is affected, this can look strikingly similar to a number of other diseases, including "brucellosis, fungal infections, septic arthritis, neoplastic disease, and osteoporosis" as well as "blastomycosis, typhoid spine, healed vertebral fractures, septic arthritis, malignant bone tumors, Paget's disease, and

ankylosing spondylitis" (Wilbur 2009, Milligan 2010). Due to the known high rate of tuberculosis in Milwaukee County around the turn of the twentieth century, observing osteological lesions caused by tuberculosis is more likely than the aforementioned fungal infections. However, in the MCIG population osteoarthritis is a significant concern in regards to confounding.

Some researchers have argued that a combination of skeletal abnormalities can sum up to a conclusion of Mtb infection, but that is also problematic (Nerlich 2009, Wilbur 2009). The ribs are often thought of as a secondary site of osseous tuberculosis replication, the vertebrae being the primary site, though lesions on the ribs may be caused from an assortment of severe immune responses that occur in the lungs (Roberts 2008). Rib lesions are therefore insufficient for identifying Mtb, even with the addition of suspected tuberculosis lesions in the vertebrae, due to the high possibility that different localities of lesions were caused from different organisms (Raff 2006). Tuberculosis has also been known to infect the weight bearing joints and the skull, most likely due to a systemic spread of the disease through the blood stream (Nerlich 2009). The ankylosing of weight bearing joints and lesions present on the skull can also be consistent with other diseases that present similarly to tuberculosis, most notably osteoarthritis, brucellosis, and Paget's disease (Nerlich 2009, Raff 2006). It is therefore difficult to say with relative certainty that an individual skeleton was infected with tuberculosis.

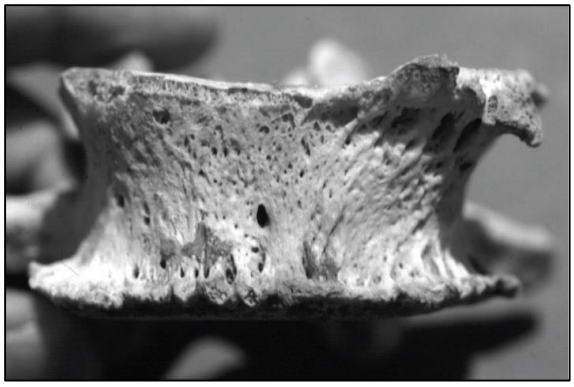

Figure 2.2 Asymmetrical lipping on the anterior aspect of the vertebral body (Nerlich 2009)

Though the lipping of the anterior aspect of vertebral bodies is very characteristic of other diseases, the large lytic lesions that tuberculosis is able to cause in the center of vertebral bodies has been argued to be unique to the disease (Taylor 1996). Osteoarthritis and syphilis in particular may present similarly to osseous tuberculosis, however Buikstra found that the articular surface lesions of Mtb generally present more asymmetrically, whereas the aforementioned diseases tend to present more symmetrically (Figure 2.2) (Buikstra and Cook 1980).

It is also important to remember how infection spreads, and how this presents itself in bone tissue. Tuberculosis is a bacterial infection, which means that it spreads via the blood stream when moving from the infected lung tissue to the hemopoietic tissues. It has been shown that tuberculosis lesions in bone will therefore follow the patterns of the vascular system (Buikstra and Cook 1980). Knowing this could help rule out metabolic disorders and tumors from tuberculosis, as the former are not restricted to the vascular pathways (Buikstra and Cook 1980).

Since van Embden's 1993 publishing of a standardized technique of tuberculosis identification, IS6110 has been the preferred gold standard of identification within the communities of paleopathology and microbiology (Roberts and Buikstra 2012, van Embden

1993). IS6110 is a repetitive element in tuberculosis DNA, meaning that the IS6110 sequence appears multiple times in the genome, occurring up to 26 times in a cell (Donoghue 2008). IS6110 is 123 base pairs in length, relatively small, making it especially useful for working with ancient DNA, which is often fragmented (Donoghue 2009). Van Embden's 1993 study used restriction enzymes to cut the sequence and southern blotting for detection (van Embden 1993). As PCR became more common in the early twenty first century, it replaced the more time consuming and costly restriction enzyme methods (Donoghue 2008). The sequence can also appear in *M. bovis* and while it can be differentiated by spoligotyping, separate PCR was carried out using the *oxyR* marker in this study.

CHAPTER THREE: HISTORICAL BACKGROUND

Tuberculosis is a disease that has a wide spatial and temporal range of infection. It can therefore be implied that most skeletal populations that predate the widespread use of antibiotics should have at least some individuals that were at one point infected, whether latently or actively. There are some populations that are more suitable for study than others due to a known high rate of infection. One of these, and the one that I focused my research on, is the Milwaukee County Institutional Grounds Cemetery.

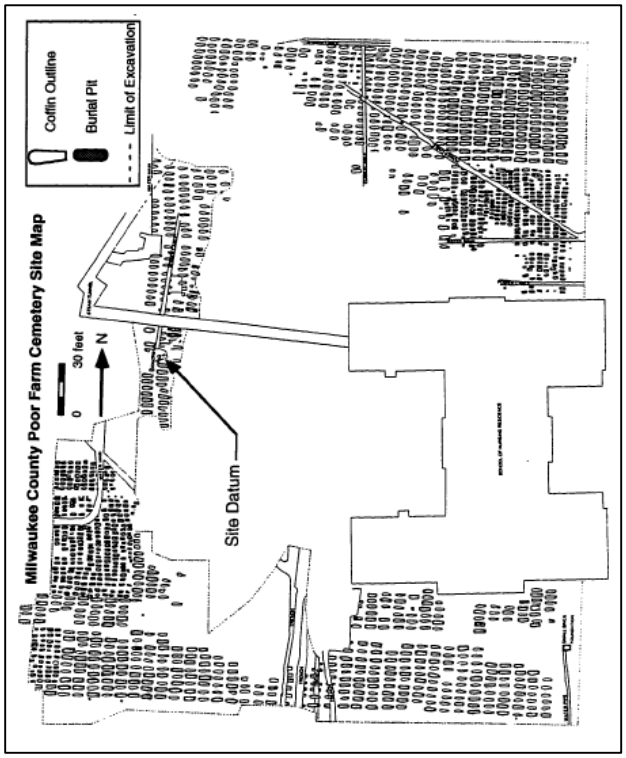

Figure 3.1 Location of excavated graves from the Milwaukee County Institution Grounds Cemetery (Richards 1997)

The Milwaukee County Institutional Grounds Cemetery (MCIGC) was part of a larger complex in Wauwatosa, Wisconsin that provided housing and burial for many of late 19th and early 20th century Milwaukee County citizens. Those interred were either unable to afford

religious burial, victims of accidents or suicides that came to the Coroner's office, or those that lived at one of the institutions on the grounds. These included a mental health facility, an orphanage, a sanitorium, and housing for the poor. A large number of burials were excavated in 1991 and 1992 by the Great Lakes Archaeological Research Center (GLARC). Burials numbered 1,061 adults and 588 sub-adults (Richards 1997). A Register of Burials was kept for the MCIG complex, recording the burials that took place between 1882 and 1974, and death certificates were recorded for both those that came from both the Coroner's office and those that died while in residence (Milligan 2010).

Milwaukee County, in the 19th and early 20th century, was marked by tuberculosis infection. Turn of the century Milwaukee was a hub for those immigrating to the Midwest, looking for work in one of Milwaukee's factories. By 1850, sixty-four percent of the population had been born abroad (Milligan 2010). This rapid influx of new citizens meant overcrowding, further sharing of limited resources, and stress on immune function that had already been lowered by travel. In the year 1900, the average number of people per dwelling in Milwaukee ranged from 5.32 to 7.32 (Leavitt 1982). Tuberculosis, a disease that spreads through droplets expelled by coughing and sneezing, had many new hosts in close proximity to infect. It is possible that the death rate of tuberculosis accounted for between ten and fourteen percent of Milwaukee deaths in the late nineteenth and early twentieth centuries. This is higher than any other single disease at the time (Leavitt 1982).

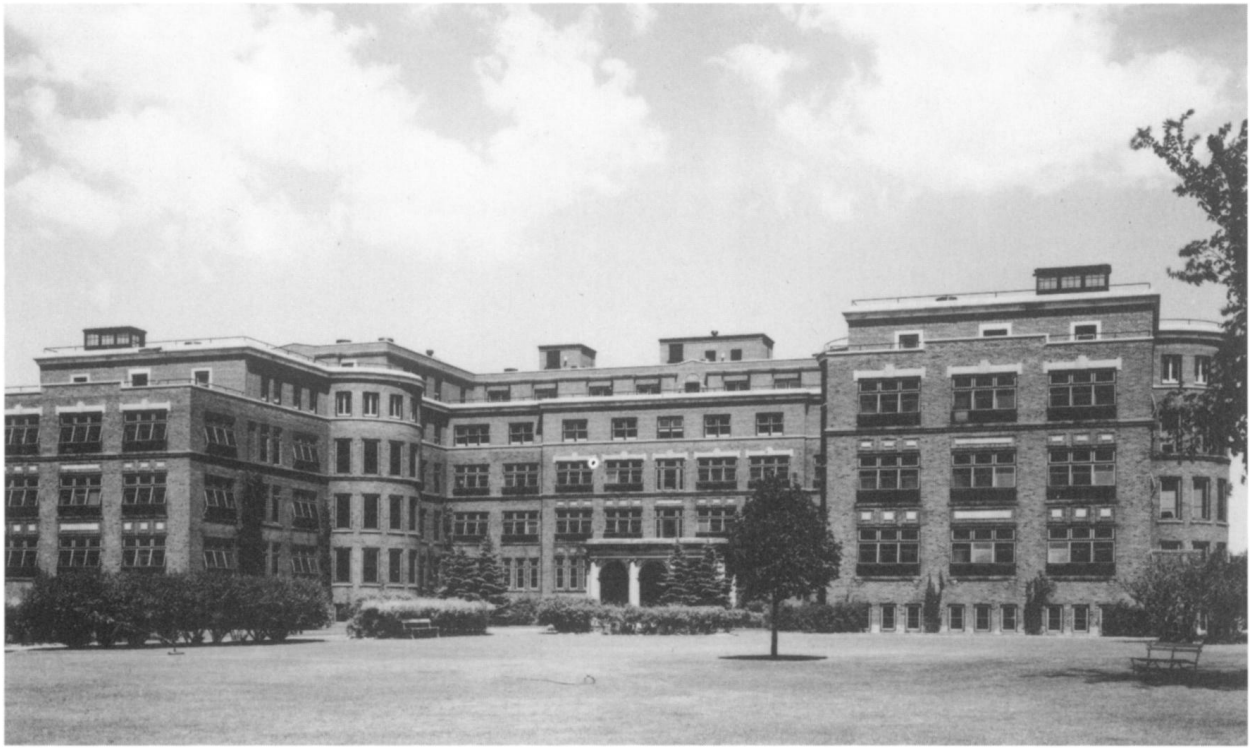

Figure 3.2 Muirdale Sanitorium (Stolder 1994)

Though tuberculosis was a large public health problem for Milwaukee County, there were limited government resources for treating the infected. There were no programs for early diagnosis, only one specialized tuberculosis hospital, and no rehabilitation facilities (Holand 1958). Unfortunately, even though in 1905 it became illegal for a physician to withhold the name of a tuberculosis patient, cases were still often left unreported. This led to the numbers of reported cases being misleadingly low and resulted in patients who could have been saved by treatment left untreated. Death benefits were often withheld from the families of consumption sufferers, and dealing with the stigma of surviving tuberculosis was too great (Stolder 1994, Leavitt 1982).

Fresh air, plenty of rest, and isolation were the prescribed treatments for a tuberculosis infection and regardless, death was often the expected result (Stolder 1994). Gradually patients were moved from home isolation into sanatoriums where they could be monitored by physicians

and isolated from the healthy population. Muirdale, in Milwaukee County, was one such institution and was situated on the Milwaukee County Institution Grounds (Richards 1997). Muirdale became the training grounds for tuberculosis nurses and doctors throughout Wisconsin (Stolder 1994). Milwaukee began a tuberculosis campaign in 1904 and in 1906 The Medical Society of Milwaukee hosted the National Association for the Study and Prevention of Tuberculosis where sputum collection cups, lung specimens, and pictures of tuberculosis patients relaxing at sanatoriums were on display for the public to see and be educated (Leavitt 1982, Stolder 1994). The state government then added a separate division to deal with tuberculosis in 1912 and finally built Muirdale Sanatorium in 1914 (Milligan 2010).

It was from this environment that the majority of the individuals represented by the 1991 and 1992 excavated burials came. The MCIG complex was originally built in order to provide indoor relief housing for Milwaukee County's poor. Over time this expanded to include housing and relief for the chronically sick, mentally ill, and government-dependent children (Richards 1997). In the early years, the conditions of the MCIG were little better than the overcrowded housing and poor sanitary conditions from which the residents came. Though a number of new buildings were added onto the complex during its years of operation, the need of the county's citizens did not lessen, and people were sent to the Milwaukee County Institution Grounds (MCIG) from as far away as Madison (Richards 1997). This meant that for those that were suffering from chronic illness, oftentimes contagious, there was little relief from the factors that made the disease spread, making the hard-worked residents prone to infection. While the crowded citizens of Milwaukee County were ravaged by *Mycobacterium tuberculosis*, the farm communities were dealing with *Mycobacterium bovis*, the bovine form of the disease. More

resources were put into controlling *M. bovis*, a program was started in 1894, as Wisconsin residents depended on the food and income from dairy and beef (Holand 1958).

It is difficult, though possible, from observing the Register of Burials to come up with an estimated number of individuals that may have died from tuberculosis disease and been buried at the Milwaukee County Institutional Grounds. Though some individuals have cause of death listed, many do not, and the process of matching the individuals to the cause of death is slow and ongoing. This is further complicated by the fact that though tuberculosis may have been officially identified by the mid-19th century, it was still colloquially referred to as “consumption” or “pthsis” for much longer, as well as often simply “pulmonary disease” (Leavitt 1982). What we do know is that there were individuals who died from tuberculosis and were buried at the MCIGC, and that the rate of tuberculosis infection for Milwaukee County between 1900 and 1920, years that the complex was in operation, was between 6.0 percent and 11.6 percent (Milligan 2010). After applying the osteological methods of tuberculosis identification to the collection, I found nine out of eighty-six skeletons that showed signs of infection. This 10.5% is consistent with the reported prevalence of infection at the time.

CHAPTER FOUR: THEORETICAL FRAMEWORK

Osteological Paradox

Wood's groundbreaking 1992 "The Osteological Paradox: Problems of Inferring Prehistoric Health from Skeletal Samples" questions the utility of inferring the health of populations from skeletal remains. If all of the people you are studying are dead, Wood wrote, then you are only seeing the subset of the population that died at that age, not those that may have survived a disease only to die of something else later in life. Wood calls this "selective mortality" and as the name implies, the population that is being studied is selective for the diseases that would show themselves on their skeleton. This means that trying to infer population prevalence from skeletal data is problematic. The sample that you have collected is over selected for the cases that were serious enough to cause death. Wood is clear that there is no easy way around this. Whenever a skeletal sample is studied, only the cases that proved fatal are the ones being observed. Wood cautions that whatever the population parameters the researcher obtains most likely overestimate the disease as it was seen in the actual population (Wood 1992).

Wood's concept of "hidden heterogeneity" takes into account the idea of the "frailty" of a population subset. For example, if there are three different populations with three different levels of frailty, the most frail will be hardest hit by the disease and will die first. The second most frail will be exposed to the disease long term and may develop skeletal infection, and the least frail will most likely survive the disease. To put this in the context of looking for tuberculosis in skeletal remains, we look for those that are in Wood's second category, those that are frail enough to have active disease, but who lived long enough to develop osseous infection. This skews the data because there could be a significant subset of individuals who had a severe

enough tuberculosis infection to be killed quickly before they could develop skeletal tuberculosis. These will most likely be in the population, but will not show any evidence of skeletal lesions. Therefore, by reporting the population parameters just from the skeletal lesions, we would be underrepresenting the amount of actual tuberculosis infections found in the population from which the individuals buried in MCIG Cemetery came (Wood 1992). This theory of Wood's can be seen in Buzon's 2005 analysis of the Legion of Honor cemetery in San Francisco, California and will be discussed later.

These two concepts would seem to be at odds with each other since one would propose that the population parameters would be overestimated, and the other would propose that they are underestimated. What Wood stresses, however, is the idea that you cannot rely on a skeletal sample to tell you about the risks of disease for a living population. He offers several different solutions to this problem. One solution would be to abandon any discussion of population parameters. A more realistic option, Wood writes, would be to continue to report population parameters, but to only compare them to other skeletal samples and to put aside the discussion of how these numbers would represent themselves in the living populations they came from. These problems are compounded when you factor in the interactions between causes of death. If someone suffered from a pulmonary disease that did not manifest skeletally, asthma for example, it could positively impact their rate of tuberculosis manifestation and progression. This means that in addition to taking into account the population parameters of a certain disease for a certain population, you also need to take into account the general health of the population and the other diseases that may have made your disease of study more pronounced.

Syndemics

In Stacie Burke's 2011 study of tuberculosis now and in the past, she discusses how the concept of Syndemics applies to tuberculosis. As originally posed by Singer in 1994, the syndemic perspective intertwines the study of bioarchaeology with social and cultural factors, such as social inequalities and structural violence (Burke 2011). The term syndemic itself refers to two or more epidemics that work synergistically to amplify the burden of disease (Singer and Clair 2003). Singer's goal was to "identify and understand the determinant interconnections among pressing health problems, sufferer and community understanding of the illness(es)/disease(s) in question, the relevant social, political, and economic forces in play, and…the environmental conditions that may have contributed to the development of ill health" (Singer and Clair 2003). Thus, a syndemic is not only the disease, but the social and cultural conditions that prevent the disease from dissipating and which sustain it in the population, doing great harm. "While the researcher may be concerned with a specific disease, such as leprosy, venereal syphilis, or tuberculosis, he cannot limit his model to that single disease" (Buikstra and Cook 1980). In the case of tuberculosis in turn of the century Milwaukee County, the tuberculosis burden was amplified by the insufficient infrastructure, the rapid rate of immigration and introduction to additional diseases, and the structural violence imposed upon the less fortunate. Additionally, Milwaukee suffered through a cholera epidemic from 1848 through 1850 and a succession of economic recessions in 1857 and 1858 (Richards 1997).

Burke uses the T cell response to disease as an example. In a study comparing the response of Caucasian Canadians to Canadian First Nations People to tuberculosis, she found that the two populations had different T cell responses to the disease. Burke postulated that this difference had arisen due to the difference in the community structure of the two populations (Burke 2011). The two populations were exposed to different pathogens throughout their evolution due to

different living and working conditions as well as the difference in their geographic origin. The Canadian First Nations People, who were traditionally hunters and gatherers, were exposed to parasitic and fungal infections present in the environment, as well as the nutritional deficiencies of their diet. Conversely, the Caucasian Canadians, who came originally from Europe, had been exposed to diseases that spread through living in close proximity to one another, such as smallpox, measles, plague, and tuberculosis (Burke 2011). This difference in exposure led the two populations to develop different immune responses to tuberculosis. Though Mtb was found in the Americas pre-European contact, due to the low population density it would have exerted a relatively low selective pressure. When the more virulent European strain of tuberculosis was introduced to the First Nations People, the effect was devastating. They did not have the immune response necessary to fight off the infection and were at an additional disadvantage due to the stress of the colonialist structure (Burke 2011).

Residents of the Milwaukee County Institutions came mostly from a low socioeconomic status and had already experienced hardship, disease, overcrowding, and other problems associated with living in a burgeoning city (Richards 1997, Milligan 2010). As well as the difficulties they had already experienced before coming to MCIG, the Institution itself presented its own share of hardships. In its early days, the poor, sick, orphans, and the insane all shared the same facility and living quarters. Even after additional facilities were built and the sick were able to be separated from the more permanent residents, they still dealt with inadequate heating, continual overcrowding, and mandatory work, which was most often hard labor (Richards 1997).

As well as the poor conditions, the inmates experienced significant structural violence by the government. Though their most basic needs were technically being met, the Almshouse was a place where Milwaukee County sent those that it did not want to deal with, thus imposing on the

residents the government's view of their place in society. There was still an underlying belief that the poor were in their situation due to a lack of "moral character" and that taking care of a society's less fortunate was the price to pay for the safety of the wealthy (Richards 1997). It would be impossible for these views to not negatively impact the Almshouse residents. Burke states in her discussion of tuberculosis and Syndemics, "To cure TB, one had to cure society" (Burke 2011).

The Legion of Honor cemetery

The Legion of Honor cemetery located in San Francisco is another late 19th century cemetery comprised of economically disadvantaged citizens who came from a population with a high immigration rate. The individuals interred were assumed to be of mostly European descent. The Legion of Honor cemetery was used as burial grounds for those that couldn't afford religious burials, smallpox victims, paupers, and medical waste. Though the MCIGC is unique in its history as both a poor farm and a burial for Milwaukee's less fortunate, there is much overlap with the Legion of Honor (LOH) cemetery in terms of the population interred. Buzon et al studied the remains in an attempt to recreate the lives of San Francisco's economically disadvantaged in the late 19th century. They found that the population was very similar to what they would expect for a turn of the century cemetery. The rates of osteoporosis, porotic hyperostosis, traumatic injuries, and tooth wear were all consistent with the three other populations the Legion of Honor cemetery was compared to. However, the remains exhibited a larger than average amount of enamel hypoplasia (Buzon 2005).

Buzon et al hypothesize that in the Legion of Honor cemetery they are seeing a unique population due to the high immigration rate. The population boom in the San Francisco bay area came from an influx of people looking for gold and then staying in the area when they did not

find the fortune they were looking for. They estimate that just 38% of residents interred were born on the West coast and that populations comprised of just such a blend of distinct cultural groups forced to live in extreme circumstances may show unique skeletal abnormalities (Buzon 2005). Buzon hypothesizes that populations exposed to extreme stressors may show a higher prevalence of certain afflictions, which agrees with Singer's 2003 study of Syndemics in which populations put under stress may show an amplification of disease. Like those interred at MCIG, the Legion of Honor population came from substandard living conditions, rapid population growth, and was battling the same infectious diseases. From historical records they know that "of the more than 25,000 people living in San Francisco in 1850, the vast majority were adult males under the age of 40" (Buzon 2005). This is again similar to MCIG, where Males make up 76.5% of the sample and 51% of the Males are of Middle age (Milligan 2010). It is interesting to note that the high rate of enamel hypoplasia for those of European descent that is reported in Buzon's study is 50%, the average is 30%, and that the MCIG males show a rate of 40.5% (Buzon 2005, Milligan 2010).

CHAPTER FIVE: METHODS

In order to test the sensitivity of the current methods of osteological identification of tuberculosis, the following steps were taken:

1) A checklist of osteological tuberculosis indicators was compiled.
2) A sample was selected from the Milwaukee County Institution Grounds cemetery.
3) The sample was analyzed for tuberculosis indicators.
4) A scraping of bone tissue was taken from the site of the lesion in those that were considered tuberculosis positive and from the healthy vertebrae from those in the sample that were considered tuberculosis negative.
5) DNA was extracted from the powdered bone tissue and was then amplified for the IS6110 repetitive element marker.
6) The amplified material was sent off to the University of Wisconsin, Madison Biotechnology Center DNA Sequencing Facility for sequencing.

Osteological indicators

Lesion patterning can differ based on sex, occupation, age, and status (Buikstra 1980). The MCIG collection comes from a mostly male population that was known to have experienced harsh conditions and heavy work load. Therefore the modern assumptions about diseases that have age or sex bias, such as osteoporosis or osteoarthritis, were not included in this analysis because it is unknown how the MCIG population compares to what we know about these biases. To compensate, I did not look at the age or sex of the individual when observing the remains in

order to keep assumptions about age and sex disease bias from my analysis. Instead, each set of remains was examined individually for pathological lesions regardless of age and sex.

As previously discussed, there are no pathognomonic indicators of osseous tuberculosis. Though there is disagreement in regards to whether or not, or with what amount of confidence, you can discern tuberculosis from other diseases, there is little argument about how osseous tuberculosis presents. Most of the current literature references Jane Buikstra and Della C. Cook's 1980 review of American palaeopathology as the most comprehensive overview of palaeopathological methods in the last thirty-five years. Buikstra and Cook discuss how the patterning of pathological lesions both within the sample and within the individual can be useful. Symmetrical patterning of lesions caused by syphilis and osteoarthritis are different from the asymmetrical nature of tuberculosis caused lesions. I reviewed osteological identification methodologies since Buikstra and Cook's 1980 work and found them in agreement in regards to the following points:

1) Asymmetrical presentation of lesions (Buikstra 1980)
2) Irregular lytic pitting (with or without complete collapse of vertebrae) (Roberts 2008, Wilbur 2008, Wilbur 2009, Nerlich 2008, Roberts 2009, Klaus 2010, Taylor 1996, Milligan 2010, Stone 2009)
3) Anterior aspect of vertebral bodies affected (Roberts 2008, Wilbur 2009, Buikstra 1980, Nerlich 2008, Milligan 2010)
4) Most likely seen in the thoracic and lumbar vertebrae (Roberts 2008, Wilbur 2009, Roberts 2009, Klaus 2010, Milligan 2010, Stone 2009)

The destructive nature of tuberculosis lesions was particularly stressed in the above literature as being characteristic of the disease above and beyond the other indicators (Wilbur 2008, Wilbur

2009, Nerlich 2008, Roberts 2009, Klaus 2010, Taylor 1996, Milligan 2010). Therefore, when analyzing the samples from the MCIG collection, I used the above four criteria to decide whether or not a skeleton showed signs of tuberculosis infection, with particular emphasis placed on the lytic nature of the lesions.

Sample

Starting with the estimate that there are approximately 100 people listed in the MCIG Register with tuberculosis or consumption listed as their cause of death, I attempted to find approximately 100 skeletons to include in my research sample. After the exclusion of sub-adults, those without a determined sex, those without vertebrae, and those without a determined age, I was left with 86 individuals.

Sub-adults were excluded for a number of reasons. Metabolic diseases are more common in sub-adults, therefore exclusion of them from the sampling would attempt to limit the number of lesions that could be mistakenly identified as tuberculosis when in actuality they were caused by a metabolic disorder. Certain diseases, tuberculosis included, are known to present differently in sub-adults and adults. Due to the difference in vascular supply and vascular patterning between the sub-adult and adult skeleton, lesions caused by pathogens often present in a different location and in a different manner (Buikstra 1980).

Skeletons that were lacking an age or sex identification were also left out of the sample as a means of practically limiting the material that needed to be analyzed. Leaving out the skeletons that had an age classification of either “Unknown” or “Ambiguous” removed 68 individuals from the analysis. The one sample that was included that had either an “Unknown” or “Ambiguous” sex determination was a specimen that had been included in Milligan’s 2010

dissertation and had been thought to show signs of tuberculosis. This same individual remained in the unknown age category.

Table 5.1 Characteristics of sample

Negative for Lesions				
	Young Adult	Middle Adult	Old Adult	Total
Female	5	4	4	13
Male	10	44	10	64
Total	15	48	14	**77**
Positive for Lesions				
	Young Adult	Middle Adult	Old Adult	Total
Female	0	1	0	1
Male	2	4	1	7
Total	2	5	1	**8**
Age ranges for the MCIG remains were established based on a multifactorial approach. Young Adult spans 20-34 years, Middle Adult spans 35-50 years, and Old Adult comprises the adults aged 50 years and above (Milligan 2010).				

The focus of my research was on osteological identification of tuberculosis from the vertebrae, which is why those skeletons lacking vertebrae were not included in my analysis. This was a significant number of individuals and after their exclusion I was left with a sample size of 86 skeletons. I then applied the osteological methods previously discussed to all 86 skeletons and found the nine of them showed evidence of tuberculosis infection according to my chosen criteria. The raw data from my collection and sampling is represented in Appendix A.

DNA extraction and PCR

Between 100-200 mg of bone powder were scraped using a sterile scalpel blade into a sterile 1.5 mL eppendorf tube from the site of the lesion. The mean weight of the collected samples was 164.73 mg and the median was 160.00 mg. Prior to collecting the bone tissue, all vertebrae were photographed. A log of the photographs is kept at the University of Wisconsin-Milwaukee Archaeological Research Laboratory. The information recorded in the MCIG Photo Log for the samples that I collected is represented in Appendix B.

An organic extraction was carried out according to the methods used in Pagan, who in 2012 carried out a study on the best method of DNA extraction from human remains in terms of yield. Though Pagan found that the most high yield method was extraction using silica beads, the organic extraction had the second highest yield and was similar to the methods carried out in Taylor, Mays, and Rimek (Pagan 2012). Despite the concern of DNA fragmentation over time, 100-200 mg was deemed sufficient due to the high percentage of guanine and cytosine present in Mycobacterium, which aid in stability (Donoghue 2004).

After collection, 700µL of TENS buffer (10mM Tris-HCl, 0.1mM EDTA, 100mM NaCl, 2% SDS, pH 8) was added to each sample tube along with 10µL Proteinase-K. The samples were vortexed vigorously and then incubated at 56° C overnight. The samples were centrifuged for three minutes the next morning at 1,500 rpm and 500µL of the supernatant was aliquoted into a sterile tube. To the supernatant, 500µL of 25:24:1 phenol/cholorform/isoamyl alcohol was added and then vortexed until it obtained a milky precipitate. This mixture was then centrifuged at 12,000 rpm for 20 minutes at room temperature. The upper aqueous phase, approximately 400µL was then transferred into a new sterile tube. To this, 500µL of 25:24:1 phenol/cholorform/isoamyl alcohol was added and then vortexed until it obtained a milky precipitate. This mixture was then centrifuged at 12,000 rpm for 20 minutes at room temperature.

The upper aqueous phase, approximately 120µL was then transferred into a new sterile tube. 40µL of sodium acetate (3M, pH 5.2) and 1100µL of -20° C 100% ethanol were added and then mixed by inversion. This was chilled at -80° C for 40 minutes before being centrifuged at 13,000 rpm for 30 minutes. The supernatant was removed and the pellet was allowed to air-dry at room temperature. After the pellet was sufficiently dry, it was then eluted in 50µL TE buffer.

Along with the extracted DNA from both the research and the control samples, one blank of deionized water to check for contamination and one positive control of the H37Rv strain of *M. tuberculosis* were included with the extraction and PCR run. The 86 samples as well as the two controls were amplified by PCR for the insertion sequence 6110 (IS6110), a known repetitive element in tuberculosis with a 123bp product. The primers for the reaction were chosen from Walker 1992, and had been used in Nerlich 2009, Rimek 2002, and Taylor 1996. The forward primer was 5'-CCTGCGAGCGTAGGCGTCGG-3' and the reverse was 5'-CTCGTCCAGCGCCGCTTCGG-3'. The samples were run following the program represented by table 5.2. All DNA extraction and PCR work was done in Trudy Turner's laboratory at the University of Wisconsin-Milwaukee.

Table 5.2 PCR program conditions

Temperature	Time	Cycles
94 C	4 minutes	1
94 C 68 C 72 C	30 seconds 1 minute 1 minute	35
72 C	3 minutes	1

The amplified DNA was sent to the DNA Sequencing Facility at the University of Wisconsin Madison. The samples were prepared in a 2:1 dilution with 6X loading dye before being run on a Sodium boric acid (SBA) gel in Sodium boric buffer with 2.2g of Agarose and 15µL of stain. The gel was run at 100 Volts for 5 minutes, then 125 Volts for 40 minutes. SBA gels were chosen for their low rate of electrolyte exhaustion, their faster running time, and their cost effectiveness (Brody 2004).

The samples that tested positive for IS6110 were then amplified for oxyR, a known repetitive element in *M. bovis* (Taylor 1996). *M. bovis*, another species that is part of the *Mycobacterium tuberculosis* complex, also has the IS6110 repetitive element marker. This makes it necessary to also amplify *M. bovis* in order to differentiate between tuberculosis and Mycobacterium in general. *M. bovis* has also been tied to extra-pulmonary forms of TB, making it more likely that it would show itself in skeletal samples (Burke 2011, Kelman 1999, Wilbur 2009).

DNA taken from the samples that tested positive for IS6110 was amplified for oxyR with the forward primer 5'-GGTGATATATCACACCATA-3' and the reverse primer 5'-CTATGCGATCAGGCGTACTTG-3' (Sreevatsan 1996). This produced a 548bp segment that was then run on a Sodium boric acid gel under the same conditions as the IS6110 product by the University of Wisconsin Madison DNA sequencing facility. None of the samples contained the oxyR marker.

Contamination control

In working with DNA, one of the biggest concerns is contamination. This is doubly important when the DNA that is being extracted is from an unstable source, such as from a skeletal collection that was interred for approximately one hundred years. Mycobacteria are

primarily environmental bacteria and can be found in the soil from which the skeletal collection was excavated (Wilbur 2009). This means that it is possible that the entire collection could show a positive IS6110 result from being interred in soil that contained Mycobacteria with the IS6110 marker, even if they were not personally infected with *M. tuberculosis.* Though the individual skeletons were cleaned after being excavated and kept in proper storage conditions, it would be naïve to assume that there would be no soil contamination. In order to address the issue of soil contamination, the scrapings of bone tissue from the vertebrae were always started superficially and were finished into the cortical bone to minimize the amount of surface area, and therefore soil contaminants, that were being collected.

The MCIG collection had been handled by a number of people between the years of 1992 when it was excavated and 2014 when I began my data collection. Most of the people that handled the remains showed good practice in handling fragile bioarchaeological material, likely did not follow proper procedure for working with material that was to be extracted for its DNA. In the 1990s, when the collection was being excavated, the United States experienced a resurgence of tuberculosis infection in hospital workers. Even with this increased incidence of tuberculosis cases, the Centers for Disease control found that the mean rate for hospital workers, the most susceptible population, was <1% (Sepkowitz 2001). This means that the likelihood that an archaeologist handling the collection was infected with tuberculosis is extremely low. Though that does not rule it out as a source of contamination, it makes the possibility of an infected archaeologist handling the remains small enough that handler contamination was not considered a risk.

Due to the large number of people who handled the remains, it was possible that the more recent DNA that had accumulated on the bones could amplify non-specifically and mask or

overpower the Mycobacterium DNA (Wilbur 2009). The assumption that modern human DNA does not pose a threat to the study of older bacterial DNA has been challenged recently due to findings that cross-contamination from other samples, from laboratory personnel, and from other animal or plant materials present, can reappear in later PCRs and can amplify non-specifically in reactions due to their more effective amplification process (Wilbur 2009).

After selection of individual remains for analysis, all further handling was compliant with NIH Guidelines for Research Involving Recombinant DNA Molecules and using Biosafety Level 1 (BL-1) practices. As well as this, the research protocols were submitted to the University of Wisconsin-Milwaukee Biosafety Program. No prior tuberculosis studies had been carried out in the facility. Though BL-1 practices were all that were required to protect lab personnel, the samples were handled using Biosafety Level 2 (BL-2) practices as a precaution against contamination. Figure 5.1 summarizes the Biosafety Level precautions for both BL-1 and BL-2.

Biosafety Level	Representative characteristics and practices*
BL-1	Standard microbiological and laboratory practices: -Decontamination of work surfaces Mechanical pipetting Hand washing after handling recombinant DNA -Food storage in specially designated areas -Appropriate protective clothing
BL-2	BL-1 practices plus: -Biosafety manual prepared and adopted for laboratories -Biological safety cabinets (I or II) for work creating aerosols -Limited access to laboratory -Hazard warning sign on door -Laboratory clothing not worn outside laboratory -Laboratory waste decontaminated -Spills and accidents reported to IBC and OBA

*These are not complete, but are intended to give a general sense of the level of containment typical of each biosafety level.

Figure 5.1 Biosafety levels and footnote from the National Institute of Health (Shipp 2003)

CHAPTER 6: RESULTS AND ANALYSIS

The intention of my work was to compare the number of tuberculosis positives from the osteological analysis to those from the DNA extraction. If there is a positive match between IS6110 positives and identification of osteological tuberculosis lesions then it can be concluded that the previous methods of tuberculosis identification on skeletal remains are useful as a tool for identifying tuberculosis. If there is not a match between IS6110 positives and identification of osteological tuberculosis lesions, then it can be concluded that he proposed osteological markers of tuberculosis are of little use. Additionally, even with a statistically significant relationship between the two variables, presence of lesions and presence of IS6110, if the error rate is too high it can still be concluded that the presence of lesions cannot accurately stand in for presence of IS6110.

The Sodium boric acid (SBA) gels were analyzed for a 123 base pair band that would be present if the skeletal material had been infected by *Mycobacterium tuberculosis*. Tuberculosis DNA was run alongside the samples and produced a bright band in the correct placement, and the negative sample that contained only deionized water did not. A 50 base pair DNA ladder from Invitrogen was also run next to the samples to provide a standard. All of the lanes showed non-specific binding and a band around the 40 base pair mark from the left-over primers.

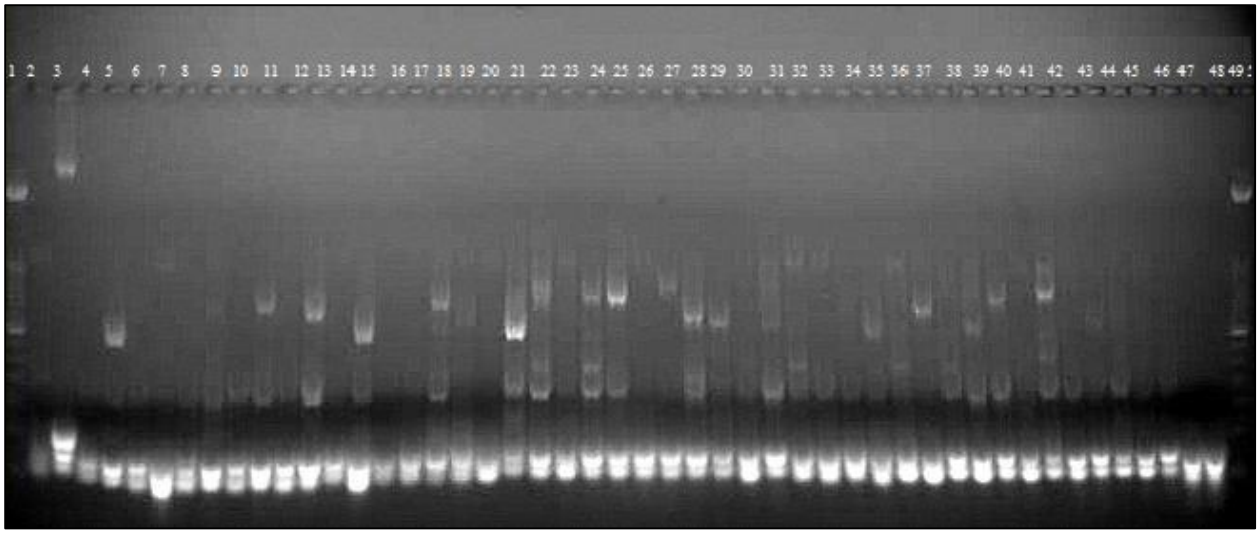

Figure 6.1 First SBA gel

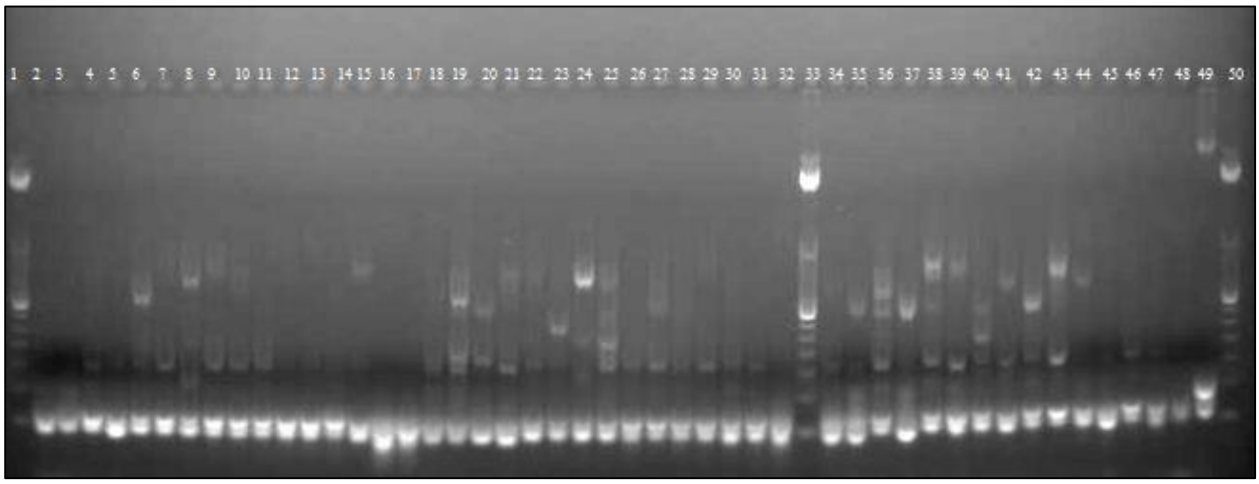

Figure 6.2 Second SBA gel

Eleven of the samples showed a band at the 123 base pair mark. All of the positive bands were significantly lighter than the positive control, which was to be expected, however, they showed significant thickening compared to the other samples. Table 6.1 provides an overview of the IS6110 positive samples. The majority of the positives were males of middle age, without lesions present. The data have a high Specificity rate of 91.09% and a low Sensitivity rate of 44.44%. Therefore, predicting IS6110 status from presence of lesions will not produce many

false positives, but it is not sensitive enough to find more than 44% of the tuberculosis positive samples.

Table 6.1 Confusion Matrix of raw data

		IS6110	
		No	Yes
Lesions	No	70	7
	Yes	5	4

Table 6.2 Confusion Matrix of predicted values using logistic regression equation

		IS6110	
		No	Yes
Lesions	No	77	0
	Yes	9	0

A logistic regression was run to test whether or not lesions can predict IS6110. Both of the variables in question are binary variables that fit with a binomial distribution. A response of "Yes" was considered a success, producing a value of one, and a response of "No" was considered a failure, producing a value of zero. From the model, a confusion matrix was created with predicted values of over 0.5 chosen as the cutoff point for the binary outcome. Though the p-value for the model was significant at 0.0076, there were no predicted responses of "Yes" for the IS6110 variable in the confusion matrix. The highest fitted value was from the predicted outcomes was 0.45. From this it can be concluded that in the Milwaukee County Institution Grounds cemetery, the presence of lesions cannot be used to predict the presence of the IS6110 repetitive element marker.

CHAPTER 7: CONCLUSIONS

Tuberculosis has been on this earth longer than humans. It has been found in some of the earliest human remains, on six of the seven continents, and is still a leading cause of death in much of the world (Clark 1987, Daniels 2006). It has even been suggested that early hominids may have come across Mycobacterium disease (Daniels 2006). Despite its long history, it was not until the last century that scientists had a reliable means of fighting the bacterium in the form of antibiotics. With the rise of DNA extraction and Polymerase Chain Reaction (PCR) as reliable detection techniques, scientists have been able to link the external skeletal manifestations of the disease with the known repetitive element marker IS6110 in the tuberculosis genome. This study has demonstrated that the presence of osteological lesions consistent with tuberculosis infection cannot be assumed to predict the presence of IS6110 in the DNA.

The skeletal remains of the Milwaukee County Institution Grounds (MCIG) cemetery had been exposed in life to a myriad of hardships and diseases, including tuberculosis. There are individuals in the register of burials with tuberculosis, consumption, or pulmonary disease listed as their cause of death. Additionally, we know that at the time the MCIG was in operation, the rate of tuberculosis infection for Milwaukee County was between 6.0 and 11.6 percent (Milligan 2010).

I began my research by reviewing the previous literature written on the osteological identification of tuberculosis. From this, I compiled a list of the techniques and main osteological indicators that are currently being applied to skeletal remains in order to assess their previous state of tuberculosis infection. Most of the current research agrees that the asymmetrical

presentation of lesions, irregular lytic pitting on the anterior aspect of thoracic or lumbar vertebral bodies and the collapse of the vertebral body can be used as indicators of tuberculosis (Buikstra 1980, Wilbur 2009, Roberts 2009, Klaus 2010, Milligan 2010, Stone 2009). After selecting a sample from the MCIG collection, I then applied the previously stated methods to the skeletal remains chosen and designated them as either having or not having evidence of tuberculosis. I chose to include the sets of remains that had both a sex and an age designation, had vertebrae present, and were adults.

After applying the osteological methods of tuberculosis identification to the collection, I found nine out of eighty-six skeletons that showed signs of infection. This 10.5% is consistent with the reported prevalence of infection at the time. Using a sterile scalpel blade, I scraped at the site of the lesions and collected between 150 and 200 milligrams of bone. For the skeletons that did not have lesions present, a scraping was taken from the body of the vertebrae. DNA from the collected bones was extracted according to the methods of Pagan, Taylor, Mays, and Rimek. The DNA was then amplified for IS6110 by PCR and the PCR products were run on a Sodium boric acid gel.

The DNA sequence selected for by the IS6110 primers is 123 base pairs (bp) in length. The positive control showed a bright band in the correct placement and the negative control, deionized water, did not. Eleven of the samples showed bands at 123 base pairs. Of the eleven samples, seven of them had also been designated as positive for tuberculosis lesions. A logistic regression was run and the data were given a binary cutoff point of 0.5 for the predicted values generated by the regression equation. Anything over the 0.5 cutoff was predicted as being positive for IS6110 using the model created from the data. The highest value generated was 0.45 which suggests that it is not possible to predict IS6110 status from the presence of vertebral

lesions in the Milwaukee County Institution Grounds cemetery. The presence of false positives within the data suggest that either IS6110 is not specific enough to *Mycobacterium tuberculosis* to continue to be used as the gold standard, as Müller's 2014 work corroborated, or that lesions in general are not specific enough to be a predictor of prior tuberculosis infection (Müller 2014).

Due to the new techniques that allow archaeologists to study ancient DNA (aDNA), much of the research on osteological tuberculosis in recent years has been focused on ancient or pre-historic populations. Most of the literature on historic sites, such as Buzon's 2005 study of the Legion of Honor cemetery, has been focused on the osteological evidence alone. Though Stone, Buikstra, Wilbur, and Nerlich have come out with recent research on the combined study of osteological and molecular biological evidence, they have focused mainly on pre-historic populations. It is possible that the natural course of bacterial evolution and the cultural and environmental influences on a population have changed drastically enough between the pre-historic and the historic time periods to affect the presentation of osteological tuberculosis.

Wood's concept of frailty

The individuals who were interred in the MCIG cemetery experienced significant hardship throughout their lives. They came from poor backgrounds where even before coming to MCIG they dealt with disease, overcrowding, malnutrition, and insufficient shelter. Once they began living at the County Institution Grounds, they experienced many of these same issues with little respite. These heinous conditions combined to produce a population that Wood would have referred to as having the highest level of frailty in his 1992 Osteological Paradox. It is possible that after an individual became infected with tuberculosis, their already weakened immune systems did not provide enough defenses for them to live long enough to produce osteological lesions, or perhaps even have time for the tuberculosis DNA to be present in their vertebrae in

any amount. This may have produced both more individuals negative for lesions, as well as negative for IS6110 who may have in life suffered from, or even died from, tuberculosis disease. Indeed, out of the nine individuals with tuberculosis or consumption listed as their cause of death in the MCIG register, three had vertebrae present and were included in my sample (Richards 1997). None of the three cases were positive for IS6110.

It is also possible, that like the Legion of Honor cemetery in San Francisco and its above average rate of enamel hypoplasia, the MCIG collection shows an unexpected pattern of tuberculosis lesions due to the uniqueness of the population, the intense conditions they lived under, and the high migration rate to the area. As Burke describes in her study of Syndemics, the entire situation of a population needs to be accounted for, not only the disease of study (Burke 2011). Besides the frailty of the population in terms of their physical health, they were also frail in regards to their status in society. The same can be said for those buried at the Legion of Honor cemetery. This may have compounded with their physical maladies to make them uniquely unsuited for carrying the burden of tuberculosis disease long enough to have it become part of their skeletal record.

Another possible explanation for why the presence of lesions does not predict for IS6110 in this population could be that IS6110 is not the gold standard for tuberculosis infection that it has long been thought to be. Müller, Roberts, and Brown recently published their findings that IS6110 can be found in ancient DNA samples that, upon sequencing, do not necessarily come from a member of the *Mycobacterium tuberculosis* Complex (MTBC), the four species of *Mycobacteria* that can potentially cause tuberculosis disease. Though the samples in this study have been tested for *Mycobacterium bovis*, one of the more common environmental pathogens of MTBC, further testing would be needed to rule out non-specific binding due to another

bacterium. This could account for the cases in this sample that showed negative for tuberculosis lesions, but tested positive for IS6110.

Future Research

The study of the Milwaukee County Institution Grounds skeletal remains is an opportunity to give voice to a unique population that had little opportunity to speak in life. This study in particular contributes to the discussion of whether or not it is possible to predict the presence of IS6110 from the skeletal remains of an historical cemetery based on osteological lesions. My conclusion is that it is not possible to predict IS6110 from skeletal lesions in this population, and that more research is required to determine the relationship between osteological lesions, tuberculosis disease, and IS6110.

As Müller recently found, IS6110 specificity may not be ideal for studies of this kind. Since van Embden published in 1993, it has been consistently used as the gold standard for tuberculosis infection. If IS6110 is present in more soil bacteria than has previously been known, then it may be time for a new gold standard to replace IS6110 in tuberculosis identification.

The goal of my study was to apply the current methods of tuberculosis identification to this skeletal population. Therefore, I used the current sampling methods to collect bone tissue (Taylor 1996, Nerlich 2008, Wilbur 2009). However, when collecting syphilis DNA from skeletal remains, the area around the lesion of interest is what is collected, not the material directly adjacent to the lesion as is the case with tuberculosis (Roberts 2008, Wilbur 2009, Roberts and Buikstra 2012). In order for this study to remain as minimally destructive as possible, I collected one sample per vertebrae and only selected from the material directly adjacent to the lesion. Further studies are needed to answer whether or not this type of sampling is sufficient for maximum collection of tuberculosis DNA.

Most of the skeletons sampled from were Middle Adult males, as that is what makes up the majority of the MCIG collection (Richards 1997, Milligan 2010). A suitable next step for research would be to focus solely on the Old Adult demographic as a means of limiting confounding. This would have the benefit of applying Wood's concept of heterogeneity to the population, as it could be theorized that those who made it to old age would be more likely to be in Wood's second level of frailty (Wood 1992).

Another possible explanation for why lesions do not predict IS6110 in the 1991 and 1992 excavated skeletal remains from the Milwaukee County Institution Grounds cemetery is that the prediction of IS6110 from lesions is not universally applicable. Much of the focus of the study of molecular identification from skeletal remains is on ancient DNA. One limiting factor associated with aDNA research is small sample sizes. This study had a sample size of 86 skeletons, which is the largest sample in the current literature. The aDNA studies that conclude that it is possible to predict lesions from IS6110 may be seeing this as an artifact of a small sample size. Additional research which applies my methods to historical cemeteries of similar sizes would be a way of testing that hypothesis.

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APPENDIX A

Sample number	Burial ID	Sex	Age	Vertebrae site scraped (g)	Lesions Present	IS6110 Present	Lane in gel	Site number on plate	Gel number
1	3039	M	Middle Age	0.1635	Yes	Yes	18	A3	1
2	2003	M	Young Adult	0.1569	Yes	Yes	19	A4	1
3	5035	F	Middle Age	0.1537	Yes	No	34	A5	1
4	5207	M	Middle Age	0.1632	Yes	No	35	A6	1
5	7230	NA	NA	0.1642	Yes	No	2	A7	2
6	9263	M	Old Age	0.1669	Yes	Yes	3	A8	2
7	1009	M	Middle Age	0.1641	Yes	Yes	18	A9	2
8	1011	F	Middle Age	0.1608	No	Yes	19	A10	2
9	1012	M	Middle Age	0.1513	Yes	No	34	A11	2
10	1013	M	Middle Age	0.1668	No	Yes	35	A12	2
11	1014	F	Old Age	0.157	No	No	4	B1	1
12	1015	M	Young Adult	0.1601	No	No	5	B2	1
13	1016	M	Middle Age	0.1758	No	No	20	B3	1
14	1017	M	Middle Age	0.1507	No	Yes	21	B4	1
15	1021	M	Middle Age	0.1519	No	No	36	B5	1
16	1023	M	Middle Age	0.16	No	No	37	B6	1
17	1024	M	Old Age	0.17	No	No	4	B7	2
18	1029	M	Young Adult	0.15	No	No	5	B8	2
19	1030	M	Middle Age	0.15	No	No	20	B9	2
20	1031	M	Middle Age	0.16	No	No	21	B10	2
21	1035	M	Young Adult	0.17	No	Yes	36	B11	2
22	1044	M	Middle Age	0.16	No	Yes	37	B12	2
23	2005	M	Middle Age	0.16	No	No	6	C1	1
24	2006	M	Young Adult	0.15	Yes	No	7	C2	1
25	2007	M	Young Adult	0.17	No	No	22	C3	1
26	2008	F	Young Adult	0.16	No	No	23	C4	1
27	2012	M	Middle Age	0.15	No	No	38	C5	1
28	2013	M	Young Adult	0.17	No	No	39	C6	1
29	2015	M	Middle Age	0.17	No	No	6	C7	2
30	2018	M	Middle Age	0.16	No	No	7	C8	2
31	2025	M	Middle Age	0.17	No	No	22	C9	2
32	2031	M	Middle Age	0.15	No	No	23	C10	2
34	2035	F	Young Adult	0.15	No	No	38	C11	2
35	2038	M	Young Adult	0.16	No	Yes	39	C12	2
36	2040	M	Middle Age	0.18	No	No	8	D1	1

37	2071	M	Young Adult	0.18	No	No	9	D2	1
38	2073	M	Old Age	0.18	No	No	24	D3	1
39	2081	M	Middle Age	0.15	No	No	25	D4	1
40	2129	F	Middle Age	0.15	No	No	40	D5	1
41	3026	M	Middle Age	0.16	No	No	41	D6	1
42	3045	M	Middle Age	0.16	No	No	8	D7	2
43	3051	M	Middle Age	0.15	No	No	9	D8	2
44	3056	M	Middle Age	0.19	No	No	24	D9	2
45	3057	F	Old Age	0.18	No	No	25	D10	2
46	3062	M	Middle Age	0.17	No	No	40	D11	2
47	3067	M	Old Age	0.15	No	No	41	D12	2
48	5002	M	Middle Age	0.15	No	No	10	E1	1
49	5020	M	Middle Age	0.16	No	No	11	E2	1
50	5092	M	Middle Age	0.15	No	No	26	E3	1
51	5095	M	Middle Age	0.19	No	No	27	E4	1
52	5101	M	Young Adult	0.2	No	No	42	E5	1
53	5107	M	Middle Age	0.17	No	No	43	E6	1
54	5134	M	Middle Age	0.18	No	No	10	E7	2
55	5166	M	Middle Age	0.16	No	No	11	E8	2
56	5186	M	Middle Age	0.15	No	No	26	E9	2
57	5190	M	Old Age	0.17	No	No	27	E10	2
58	5211	M	Middle Age	0.16	No	No	42	E11	2
59	5228	M	Old Age	0.16	No	No	43	E12	2
60	5235	M	Middle Age	0.15	No	No	12	F1	1
61	5238	F	Old Age	0.15	No	No	13	F2	1
62	7042	M	Old Age	0.16	No	No	28	F3	1
63	7070	F	Young Adult	0.15	No	No	29	F4	1
64	7098	F	Old Age	0.17	No	No	44	F5	1
66	7172	F	Middle Age	0.17	No	No	45	F6	1
67	7185	M	Middle Age	0.2	No	No	12	F7	2
68	8016	M	Young Adult	0.17	No	No	13	F8	2
69	8017	M	Middle Age	0.17	No	No	28	F9	2
70	8043	M	Middle Age	0.18	No	No	29	F10	2
71	8065	M	Middle Age	0.16	No	No	44	F11	2
72	8069	M	Middle Age	0.16	No	No	45	F12	2
73	8074	M	Middle Age	0.17	No	No	14	G1	1
74	8100	M	Old Age	0.17	No	No	15	G2	1
75	8120	M	Middle Age	0.18	No	No	30	G3	1
76	8174	M	Middle Age	0.2	No	No	31	G4	1
78	9215	M	Old Age	0.15	No	No	46	G5	1
79	9245	M	Old Age	0.15	No	No	47	G6	1

80	9263	M	Old Age	0.17	No	No	14	G7	2
81	9280	M	Middle Age	0.19	No	No	15	G8	2
82	9291	M	Young Adult	0.16	No	Yes	30	G9	2
83	9309	M	Middle Age	0.15	No	No	31	G10	2
84	9314	F	Young Adult	0.16	No	No	46	G11	2
85	9315	F	Middle Age	0.2	No	No	47	G12	2
86	9317	M	Middle Age	0.2	No	No	16	H1	1
87	9333	F	Young Adult	0.18	No	No	17	H2	1
88	9344	M	Middle Age	0.15	No	No	32	H3	1
89	9346	M	Middle Age	0.15	No	No	33	H4	1

APPENDIX B

Frame	DSCN #:	Subject	Lot Number	Initials	Date
1	15	Vertebra	3039	CRJ	3/7/14
2	16	Vertebra	3039	CRJ	3/7/14
3	17	Vertebra	2003	CRJ	3/7/14
4	18	Vertebra	2003	CRJ	3/7/14
5	19	Vertebra	5035	HMW	3/7/14
6	20	Vertebra	5207	HMW	3/7/14
7	21	Vertebra	7230	HMW	3/7/14
8	22	Vertebra	9263	HMW	3/7/14
9	36	Vertebra	1009	HMW	3/7/14
10	37	Vertebra	1011	HMW	3/7/14
11	69	Vertebra	5190	HMW	5/7/14
12	70	Vertebra	5190	HMW	5/7/14
13	71	Vertebra	1029	HMW	4/2/14
14	72	Vertebra	1030	HMW	4/2/14
15	73	Vertebra	1031	HMW	4/2/14
16	74	Vertebra	1035	HMW	4/2/14
17	75	Vertebra	1044	HMW	4/2/14
18	76	Vertebra	2005	HMW	4/2/14
19	77	Vertebra	5211	HMW	5/7/14
20	78	Vertebra	5211	HMW	5/7/14
21	84	Vertebra	5228	HMW	5/7/14
22	85	Vertebra	5228	HMW	5/7/14
23	86	Vertebra	5092	HMW	4/4/14
24	87	Vertebra	5092	HMW	4/4/14
25	88	Vertebra	5235	HMW	5/7/14
26	89	Vertebra	5235	HMW	5/7/14
27	90	Vertebra	5095	HMW	4/4/14
28	91	Vertebra	5095	HMW	4/4/14
29	92	Vertebra	5238	HMW	5/7/14
30	93	Vertebra	5238	HMW	5/7/14
31	94	Vertebra	5101	HMW	4/4/14

32	95	Vertebra	5101	HMW	4/4/14
33	96	Vertebra	5107	HMW	4/4/14
34	97	Vertebra	5107	HMW	4/4/14
35	98	Vertebra	7042	HMW	5/7/14
36	99	Vertebra	7042	HMW	5/7/14
37	100	Vertebra	7070	HMW	5/7/14
38	101	Vertebra	7070	HMW	5/7/14
39	102	Vertebra	7098	HMW	5/7/14
40	103	Vertebra	7098	HMW	5/7/14
41	104	Vertebra	1012	HMW	4/11/14
42	105	Vertebra	7172	HMW	5/7/14
43	106	Vertebra	7172	HMW	5/7/14
44	107	Vertebra	7185	HMW	5/7/14
45	108	Vertebra	7185	HMW	5/7/14
46	109	Vertebra	8016	HMW	5/7/14
47	110	Vertebra	8016	HMW	5/7/14
48	111	Vertebra	8017	HMW	5/7/14
49	112	Vertebra	8017	HMW	5/7/14
50	113	Vertebra	8043	HMW	5/7/14
51	114	Vertebra	8043	HMW	5/7/14
52	115	Vertebra	8065	HMW	5/7/14
53	116	Vertebra	1013	HMW	4/11/14
54	117	Vertebra	1014	HMW	4/11/14
55	118	Vertebra	1015	HMW	4/11/14
56	119	Vertebra	8065	HMW	5/7/14
57	120	Vertebra	8069	HMW	5/7/14
58	121	Vertebra	8069	HMW	5/7/14
59	122	Vertebra	1016	HMW	4/16/14
60	123	Vertebra	1017	HMW	4/16/14
61	124	Vertebra	8074	HMW	5/7/14
62	125	Vertebra	8074	HMW	5/7/14
63	126	Vertebra	8100	HMW	5/7/14
64	127	Vertebra	1021	HMW	4/16/14
65	128	Vertebra	8100	HMW	5/7/14
66	129	Vertebra	8120	HMW	5/7/14
67	130	Vertebra	8120	HMW	5/7/14
68	131	Vertebra	8174	HMW	5/7/14
69	132	Vertebra	8174	HMW	5/7/14
70	133	Vertebra	9215	HMW	5/7/14
71	134	Vertebra	1023	HMW	4/16/14

72	135	Vertebra	9215	HMW	5/7/14
73	136	Vertebra	9245	HMW	5/7/14
74	137	Vertebra	9245	HMW	5/7/14
75	138	Vertebra	9263	HMW	5/7/14
76	139	Vertebra	1024	HMW	4/16/14
77	140	Vertebra	3057	HMW	4/30/14
78	141	Vertebra	3062	HMW	4/30/14
79	142	Vertebra	3062	HMW	4/30/14
80	143	Vertebra	9263	HMW	5/7/14
81	144	Vertebra	3067	HMW	4/30/14
82	145	Vertebra	3067	HMW	4/30/14
83	146	Vertebra	5002	HMW	4/30/14
84	147	Vertebra	5002	HMW	4/30/14
85	148	Vertebra	5020	HMW	4/30/14
86	149	Vertebra	5020	HMW	4/30/14
87	150	Vertebra	5134	HMW	5/2/14
88	151	Vertebra	5134	HMW	5/2/14
89	152	Vertebra	5166	HMW	5/2/14
90	153	Vertebra	5166	HMW	5/2/14
91	154	Vertebra	9280	HMW	5/7/14
92	155	Vertebra	9280	HMW	5/7/14
93	156	Vertebra	9291	HMW	5/7/14
94	157	Vertebra	9291	HMW	5/7/14
95	158	Vertebra	9309	HMW	5/7/14
96	159	Vertebra	9309	HMW	5/7/14
97	160	Vertebra	5186	HMW	5/2/14
98	161	Vertebra	5186	HMW	5/2/14
99	162	Vertebra	9314	HMW	5/7/14
100	163	Vertebra	9314	HMW	5/7/14
101	164	Vertebra	9315	HMW	5/7/14
102	165	Vertebra	9315	HMW	5/7/14
103	166	Vertebra	9317	HMW	5/7/14
104	167	Vertebra	9317	HMW	5/7/14
105	168	Vertebra	9333	HMW	5/7/14
106	169	Vertebra	9333	HMW	5/7/14
107	170	Vertebra	9344	HMW	5/7/14
108	171	Vertebra	9344	HMW	5/7/14
109	172	Vertebra	9346	HMW	5/7/14
110	173	Vertebra	9346	HMW	5/7/14
111	814	Vertebra	2005	HMW	4/23/14
112	815	Vertebra	2006	HMW	4/23/14

113	816	Vertebra	2006	HMW	4/23/14
114	817	Vertebra	2007	HMW	4/23/14
115	818	Vertebra	2007	HMW	4/23/14
116	819	Vertebra	2008	HMW	4/23/14
117	820	Vertebra	2008	HMW	4/23/14
118	821	Vertebra	2012	HMW	4/23/14
119	822	Vertebra	2012	HMW	4/23/14
120	823	Vertebra	2013	HMW	4/23/14
121	824	Vertebra	2013	HMW	4/23/14
122	825	Vertebra	2015	HMW	4/23/14
123	826	Vertebra	2015	HMW	4/23/14
124	827	Vertebra	2018	HMW	4/23/14
125	828	Vertebra	2018	HMW	4/23/14
126	829	Vertebra	2025	HMW	4/23/14
127	830	Vertebra	2025	HMW	4/25/14
128	831	Vertebra	2031	HMW	4/25/14
129	832	Vertebra	2031	HMW	4/25/14
130	833	Vertebra	2035	HMW	4/25/14
131	834	Vertebra	2035	HMW	4/25/14
132	835	Vertebra	2038	HMW	4/25/14
133	836	Vertebra	2038	HMW	4/25/14
134	837	Vertebra	2040	HMW	4/25/14
135	838	Vertebra	2040	HMW	4/25/14
136	839	Vertebra	2071	HMW	4/25/14
137	840	Vertebra	2071	HMW	4/25/14
138	841	Vertebra	2073	HMW	4/25/14
139	842	Vertebra	2073	HMW	4/25/14
140	843	Vertebra	2081	HMW	4/25/14
141	844	Vertebra	2081	HMW	4/25/14
142	845	Vertebra	2129	HMW	4/25/14
143	846	Vertebra	2129	HMW	4/25/14
144	847	Vertebra	3026	HMW	4/25/14
145	848	Vertebra	3026	HMW	4/25/14
146	849	Vertebra	3045	HMW	4/25/14
147	850	Vertebra	3045	HMW	4/25/14
148	851	Vertebra	3051	HMW	4/25/14
149	852	Vertebra	3051	HMW	4/25/14
150	853	Vertebra	3056	HMW	4/25/14
151	854	Vertebra	3056	HMW	4/25/14
152	855	Vertebra	3057	HMW	4/25/14