A Critical Evaluation of DNA analysis for Palaeopathological Research

Corrie Hyland

Abstract
DNA analysis is a recent analytical technique to evaluate ancient diseases. Paleopathological research has used ancient DNA analysis to differentially diagnose pathological lesions, explore the origins and spread of disease, and better understand how diseases have interacted in past populations. This paper critically evaluates the challenges of ancient DNA research including the ethics of destructive analysis, the viability of analysis due to DNA preservation, and concerns of contamination. Despite these challenges ancient DNA analysis provides novel data for the discipline of paleopathology and ancient DNA research is worthy of further consideration.

Keywords
Palaeopathology — Bioarcheology — DNA Analysis

1. Introduction

Technological advancements have greatly benefitted the field of paleopathology. Microscopy and radiography have allowed researchers to analyse skeletal lesions in new ways to extract unique information. DNA analysis is the most recent of these new technologies which expands the research potential of paleopathological studies. A wide variety of research questions can now be answered using this analytical tool. Paleopathological research has been used to differentially diagnose pathological lesions, explore the origins and spread of disease, and better understand how diseases have interacted in past populations. Ancient DNA analysis comes with additional challenges which must be evaluated before analysis is undertaken, monitored, and properly documented: the ethics of destructive analysis, the viability of analysis due to DNA preservation, and concerns of contamination. This paper will introduce readers to the basic mechanics of DNA analysis, critically evaluate its use in paleopathological research, and argue that this technique can provide novel data for the discipline if ethical, preservation, and contamination factors are accounted for.

2. Background of ancient DNA analysis

DNA analysis has experienced exponential growth in the precision, accuracy, and quality of information it provides. The major components of DNA analysis include DNA extraction, amplification, targeting, and sequencing (Smalla et al., 1993). While DNA extraction methods have been individually developed to extract DNA from specific sample types, there are four major steps which all extraction methods must include (Smalla et al., 1993; Tan and Yiap, 2009). The sample tissues are first broken apart to expose individual cells or cell groups and cellular proteins are denatured to release the DNA from within the cell membrane(s) or wall (Tan and Yiap, 2009). The DNA is then unwound using the appropriate nuclease, and the unwound DNA is separated from the other cellular components and possible contaminants (Tan and Yiap, 2009). Another major advancement in ancient DNA analysis was the use of polymerase chain reaction (PCR) to successfully amplify ancient DNA from bone samples in 1989 (Hagelberg and Sykes, 1989; Rafi et al., 1994). Amplification of short strands of degraded ancient DNA was made possible through the repeated synthesis and denaturing of the targeted DNA segments using the protein polymerase coupled with heat cycles (Saiki et al., 1988). The PCR technique improved the targeting ability, DNA length, and yield for ancient DNA extraction (Saiki et al., 1988). This resulted in smaller required sample sizes and the ability to target a wide range of ancient DNA segments (Saiki et al., 1988). DNA targeting occurs simultaneously with DNA amplification during PCR, where two techniques are commonly employed: direct sequencing and cloning PCR. These targeting methods vary based on the number of PCR amplifications, which are performed before sequencing (Roberts and Ingham, 2008). The direct method involves fewer amplifications, is more specific in targeting, and is less costly compared to the cloning method which allows researchers to verify sequences (Roberts and Ingham, 2008). Sequencing, the final stage of DNA analysis, allows researchers to analyse the DNA code (Roberts and Ingham, 2008). Sanger sequencing, through the use of gel electrophoresis, has historically been used to identify the order of the nucleotide base pairs in DNA, but developments of next-generation sequencing methods, such as real-time parallel

sequencing, have become the preferred method due to their reduced time and cost (Shendure and Ji, 2008). The development of appropriate extraction, amplification, targeting, and sequencing of DNA has vastly improved researchers' ability to perform ancient DNA analysis (Roberts and Ingham, 2008).

Spoligotyping is an important analytical technique of DNA analysis for paleopathological research which can identify DNA sources to the subspecies level (Mays et al., 2001; Zink et al., 2004). This subspecies identification is based on characteristic direct repeat regions which form a unique spoligotyping signature (Mays et al., 2001; Zink et al., 2004). Direct repeat (DR) regions are sections of repeating DNA that are 36 base pairs long and interspersed by nonrepetitive DNA spacers (Mays et al., 2001). The sample DR regions are compared to an international database of contemporary clinically-derived DR regions to identify the subspecies of various pathogens. For example, *Mycobacterium bovis* was differentiated from *Mycobacterium tuberculosis* based on the lack of terminal five spacers at the 3' end of the DR region (Mays et al., 2001). Spoligotyping can be used to differentiate subspecies of ancient pathogens. However, the recovery of spoligotyping signatures decreases with the age of the sample (Zink et al., 2004). Overall, this analytical technique has allowed researchers to differentiate between the potential subspecies of disease-causing pathogens.

DNA analysis can be performed on any biological material containing cells, but certain sample types may be better suited for ancient pathological DNA analysis than others. Bone, teeth, soft tissues, hairs, and coprolites have all been used for DNA studies in areas of biological anthropology (O'Rourke et al., 2000). Due to the age of the samples, bone is often used for DNA analysis of ancient remains because the DNA binds to the hydroxyapatite within the bony matrix, which protects it from degradation (O'Rourke et al., 2000). The best extraction methods for bone samples is to drill long bones, or use small fragments, because the spongy trabecular bone yields 20 times more DNA than compact bone (O'Rourke, 2000). It is recommended that lesions should not be directly sampled due to the higher possibility of contamination within the lesions (O'Rourke, 2000). Teeth are another sample type for DNA analysis and have the advantage of providing multiple samples from the same individual (Haak et al., 2008; O'Rourke, 2000). Sampled teeth should be unerupted and without caries to decrease the chance of contamination, and sampling methods should include powdering or sectioning to allow for the teeth to be reconstructed (O'Rourke, 2000). Soft tissues, such as mummified remains, should be sampled from desiccated tissues and be subsurface sampled to decrease contamination (Zink et al., 2003; O'Rourke et al., 2000). Due to their lower porosity, hair samples are believed to have contamination resistant properties when compared to bone and soft tissues. Hair is thus a potential sample type when contamination is a concern (Anastasiou and Mitchell, 2013). Coprolites have been used as a viable source of parasitic eggs for DNA analysis (Gilbert et al., 2007; O'Rourke et al., 2000). Bone, teeth, soft tissues, hairs, and coprolites are all suitable DNA sample types, although the choice of sample will depend on the context of research and the concern for contamination.

3. Applications of DNA analysis to Paleopathological research

The use of DNA analysis for the study of ancient diseases first began in the 1990s with the detection of *Mycobacterium tuberculosis* (Spigelman and Lemma, 1993). Since that time, the use of DNA analysis has expanded to include differentially diagnosing diseases, investigating the genomic origins of infectious agents, studying the origins, spread of disease, and understanding how diseases interacted in the past (O'Rourke et al., 2000; Roberts and Ingham, 2008). The use of DNA analysis in paleopathology will allow researchers to discover information which was previously unavailable and has provided opportunities for new fields of research.

DNA analysis has been used to differentially diagnose skeletal remains whose pathological lesions were non-characteristic. The spoligotyping technique was developed to differentiate between *M. tuberculosis* and *M. bovis*, which was traditionally challenging to differentially diagnose based on a macroscopic examination of lesions (Kamerbeek et al., 1997). The two sub-strains were differentiated by analysing direct repeat regions and comparing the end segments (Kamerbeek et al., 1997). This technique has been widely used to differentiate the pathogenic causes of . Spoligotyping as well as targeting M. tuberculosis-specific gene sequences (including mtp40, oxyR, and IS6110) was conducted for remains from Wharram Percy (Mays et al., 2001). This study found a similar lack of *M. bovis* and brucellosis DNA in analysed individuals. Another study used DNA analysis to assist in the differential diagnosis of tuberculosis for seven individuals who displayed visceral surface rib lesions (Mays et al., 2002). The results revealed only one positive identification of *M. tuberculosis* (Mays et al., 2002). This was used as DNA evidence to conclude that visceral rib lesions may not be used to differentially diagnose tuberculosis in skeletal remains (Mays et al., 2002). In this way, DNA analysis was used to strengthen macroscopic differential diagnosis techniques (Mays et al., 2002). DNA analysis was successfully used to differentially diagnose leprosy in one of the two analysed individuals who displayed rhinomaxillary syndrome (Likovsky et al., 2006). This positive DNA result of *Mycrobacterium leprae* was used to support the presence of leprosy in Bohemia before the Crusades (Likovsky et al., 2006). DNA analysis may be one of the only methods to identify diseases which leave no skeletal trace including Chagas' disease (Guhl et al., 1999; O'Rourke et al., 2000). Chagas' disease was recognized in mummified human remains by using DNA analysis to identify *Trypanosoma cruzi* (Guhl et al., 1999). The positive identification allowed the researchers to differentially diagnose Chagas's

disease in the absence of pathological changes (Guhl et al., 1999). Differential diagnosis is one of the primary uses of DNA analytical techniques in paleopathology.

Genomic DNA analyses of both ancient and modern pathogens can be used to determine the evolutionary origins and spread of infectious agents. The origins of leprosy have been determined through genomic studies of *M. leprae* DNA. Comparative DNA analysis of *M. leprae* and *M. tuberculosis* revealed that half of the *M. leprae* genome was occupied by pseudogenes which indicated a downsizing genome (Monot et al., 2005). Additionally, 175 clinical and laboratory single-nucleotide polymorphisms of *M. leprae* from 21 countries were compared to determine the area of origin and spread of leprosy (Monot et al., 2005). The results of these comparisons determined that leprosy was likely introduced into West Africa from European or North African interactions. The West African strain was then spread to the Caribbean islands and Brazil during the 18th-century slave trades, and the North American strain was likely introduced through colonial migrations from the Old World (Monot et al., 2005). While Leprosy has been detected in armadillo populations, its global transmission can be attributed to human interactions and mass migration events (Kirchheimer and Storks 1971; Monot et al., 2005). The characterization of ancient pathogen DNA provides time-stamped genomes which researchers use to calibrate the molecular clocks of modern pathogen genomes and create more accurate evolutionary models (Harkins and Stone, 2015). This additional information will allow for fine grain analysis, and future research is predicted to involve ancient population-level studies and the co-evolutionary history of humans and pathogens (Harkins and Stone, 2015). Genomic studies of ancient pathological DNA are a growing area of research which promises to provide a better understanding of disease origin and spread.

The study of the origin and spread of disease may also be undertaken through the identification of disease in early time periods and unique geographic areas. 9000-year-old skeletal remains were discovered in 2008 which displayed pathological lesions suggestive of tuberculosis (Hershkovitz et al., 2008). The preservation of the remains in an anaerobic environment assisted in the long-term DNA preservation and the successful analysis of *M. tuberculosis* DNA (Hershkovitz et al., 2008). Within this analysis lipid biomarkers were used to identify the signature of *M. tuberculosis* cell walls which further supported the differential diagnosis of Tuberculosis (Hershkovitz et al., 2008). Remains from a variety of global contexts were analysed using spoligotyping to identify the spread and history of *M. tuberculosis* and *M. bovis* (Zink et al., 2004). The authors found that none of the human samples analysed were infected by *M. bovis* (Zink et al., 2004). This evidence was further used to support the evolution of *M. tuberculosis* from an ancestral strain (Zink et al., 2004). The history of Lyme disease was clarified through the DNA analysis of ticks from museum deer furs, which were analysed for spirochete specific DNA sequences (Persing et al., 1990). The identification of Borrelia burgdorferi in the historic pelts identified the presence of Lyme disease in North America 10 years earlier than the first human reported cases (Persing et al., 1990). A positive identification of pathogen DNA of unique temporal or geographic context provides important information about the origins and spread of disease.

DNA analysis can reveal how past diseases interacted within the human population. DNA analysis was used to identify the coinfection of individuals with both leprosy and tuberculosis (Donoghue et al., 2005). At least 22 individuals, some displaying pathological lesions for either tuberculosis or leprosy and some with no lesions, were sampled from various global contexts and time periods (Donoghue et al., 2005). The remains were independently analysed in two laboratories to verify the results, and while the results from the two laboratories varied, both laboratories found evidence of coinfection in the sampled individuals (Donoghue et al., 2005). These results were used to discredit the co-resistance hypothesis, which states that infection with one of the diseases provides immunity for the other (Donoghue et al., 2005). This study also provided strong supportive evidence that both tuberculosis and leprosy co-existed in past populations (Donoghue et al., 2005). Using DNA analysis, future co-infection research will be possible to identify how past diseases interacted with each other and within human populations . DNA analysis has supported paleopathologists working in traditional areas of research and created new opportunities to explore previously unanswered questions. DNA evidence has been used in paleopathology to support differential diagnoses, explore the origins, and spread of disease, and provide insight into disease interactions (O'Rourke et al., 2000; Roberts and Ingham, 2008). Nonetheless, paleopathological DNA analysis is not a perfect analytical tool, and attention must be paid to the ethical concerns of a destructive analysis, as well as the limitations caused by DNA preservation, and the impacts of contamination (Bouwman and Brown, 2004; Mays et al., 2001; O'Rourke et al., 2000; Roberts and Ingham, 2008). The quality of paleopathological research will grow by acknowledging and documenting these challenges in publications.

4. Critical evaluation of the use of DNA analysis for Paleopathological research

Ancient DNA analysis is complicated by the ethical concerns of the destructive nature of this analysis. As previously described, DNA analysis requires the direct sampling and destruction of sample material to analyse the sample's DNA signature. This destructive analysis becomes ethically questionable when researchers are analysing limited historical resources such as archaeological artifacts and human remains (Roberts and Ingham, 2008). The destruction of human remains also raises ethical questions about the personal integrity of the human remains, the value of individualism, and the

unethical treatment of the dead (Kaufmann and Ruhli, 2010). Additionally, the religious affiliation of the deceased and their descendants may disagree with posthumorous medical research, and descendant groups may request that the results of certain forms of analysis be known only by certain individuals (Kaufmann and Ruhli, 2010). In certain situations, the goals of the paleopathologist may not match ethical expectations, and so compromises must be made. For instance, many countries have created legislation which returns the control of human remains to the likely descendent populations (Mays, 2010). Archaeologists should familiarize themselves with the local laws, and speak with potential community shareholders before conducting any destructive analyses on human remains (O'Rourke et al., 2000). Advances in DNA analysis have made these procedures much less invasive, and the required sample size has been substantially reduced by using PCR techniques (O'Rourke et al., 2000; Wayne et al., 1999:459). Due to the destructive nature of DNA analysis, these techniques should only be used to answer questions that cannot be answered through non-destructive methods (Roberts and Ingham, 2008).

Preservation is another concern of ancient DNA analysis which accounts for one of the major challenges of performing pathogen DNA research. DNA preservation is impacted by the burial environment, the age of the sample, and post-excavation activities (O'Rourke et al., 2000; Roberts and Ingham, 2008; Wayne et al., 1999). Methods exist which determine the level of preservation, allow researchers to modify their analytical techniques and further enhance the extraction of ancient DNA (Mays et al., 2001; Roberts and Ingham, 2008). As we shall see, the unique lifecycle of pathogens also impacts DNA preservation.

The burial environment is the most important factor in determining the levels of preservation and degradation. For instance, low-temperature environments assist in ancient DNA preservation; a decrease of 20 degrees Celsius corresponds to a decrease in base degradation of 10-25 times the normal rate (O'Rourke et al., 2000). Environmental factors such as low moisture levels, moderate pH, and UV protection will also increase DNA preservation within samples and result in higher DNA yields (Wayne et al., 1999; Zink et al., 2004). Remains found in high altitude, cold, dry caves, or within arctic permafrost are likely to provide optimal preservation and result in higher yields (Wayne et al., 1999). Environments with variable temperatures and moderate levels of humidity may make DNA preservation impossible over long periods of time (von Hunnius et al., 2007). The environmental conditions of the source material should thus be critically evaluated to determine the feasibility of DNA preservation.

The age of sample material is negatively correlated with ancient DNA preservation. Even in ideal burial environments, advanced sample age will result in molecular degradation of DNA and make analysis challenging or impossible (O'Rourke et al., 2000). Hydrolysis or oxidation may degrade nucleic acids over time, and result in an upper limit of 100,000 years for DNA analysis (Wayne et al., 1999; Zink et al., 2004). Samples older than 10,000 years are unlikely to contain DNA. This time frame can be further reduced depending on the size of the target DNA segment (O'Rourke et al., 2000). DNA segments less than 300-500 base pairs long have limited analytical lifespans of less than 10,000 years due to the increased susceptibility of large DNA strands to damages (O'Rourke et al., 2000). In short, paleopathological DNA analysis is limited to more recent timescales due to changes in preservation.

Post-excavation activities will play a role in ancient DNA preservation and should be critically evaluated as a part of the excavation strategy. Post-excavation activities can simultaneously reduce the amount of endogenous DNA (DNA from within the sample) and introduce exogenous DNA (contaminating DNA from sources outside of the sample) to the sample (Roberts and Ingham, 2008). Most of the damage to archaeological sample DNA occurs immediately post-mortem, but the storage conditions of the remains can also impact preservation (O'Rourke et al., 2000; Roberts and Ingham, 2008). Fresh, unwashed, recently excavated bone was found to have more authentic DNA and preservation levels six times higher than museum stored bones (Roberts and Ingham, 2008). High energy paleopathological analyses may also impact DNA preservation, including radiography which may decrease the quality of the DNA due to the technique's use of high energy x-rays (Roberts and Ingham, 2008). Therefore, the post-excavation history of all samples should be considered before conducting DNA analysis and this information should be included in all publications.

There are methods which researchers can use to measure the relative level of DNA preservation, which may be impacted by factors such as environment, sample age, and post-excavation activities. Researchers can determine if low levels of mitochondrial DNA (mtDNA) are the result of poor overall preservation by comparing the preservation of the human nuclear DNA to mtDNA (Roberts and Ingham, 2008). If both nuclear and mtDNA preservation is low, then environmental or post-excavation strategies are the likely cause of poor preservation (Roberts and Ingham, 2008). Biochemical markers, such as residues and changes in amino acids, can be analysed as an indirect representation of preservation (Roberts and Ingham, 2008). Researchers can analyse associated archaeofaunal remains to determine if their preservation matches the human samples and indicates poor site conditions (Roberts and Ingham, 2008). Research designs can be modified to maximize DNA yields by targeting short DNA fragments, which are more likely to survive degradation compared to longer strands (Mays et al., 2001; O'Rourke et al., 2000; Roberts and Ingham, 2008). Despite the challenges of DNA preservation, researchers can extract significant quantities of endogenous DNA by critically evaluating the burial environment, sample age, post-excavation strategies and analytical techniques and adjusting the research methodology appropriately.

Paleopathological DNA analysis is complicated by the additional preservation requirements of analysing pathogen DNA which is sampled from human and faunal remains. Treponemal diseases fall into this consideration because they do not meet the requirements for DNA preservation in archaeological materials (Bouwman and Brown, 2005; von Hunnius et al., 2007). Treponemal diseases are a bacterial disease characterized by the spiral form of the bacterial agent, and include venereal syphilis (Bouwman and Brown, 2004). To successfully analyse ancient pathological DNA, the pathogen must be incorporated into the bone material by either remodelling during the latter stages of the disease (this occurs for both tuberculosis and leprosy) or be a blood-borne pathogen which is transferred to the bone matrix after death (this is the case for Plasmodium) (Bouwman and Brown, 2005). Venereal syphilis, Treponoma pallidum, does not preserve in archaeological bone because the pathogen is not present in large numbers during the tertiary stage in which the bone is remodeled (Bouwman and Brown, 2005). The pathogen load is at significantly low levels during this phase such that clinical patients are considered non-contagious (von Hunnius et al., 2007). The Secondary phase in which Treponema pallidum subsp. pallidum is at its highest loads does not involve bone remodeling and results in bones without pathological lesions having large amounts of pathogenic DNA (Bouwman and Brown, 2005). The lack of syphilitic DNA preservation, has been supported by research of venereal syphilis DNA preservation in rabbits (von Hunnius et al., 2007). The bone remains from rabbits at both the secondary and tertiary stage of venereal syphilis were analysed for treponemal DNA (von Hunnius et al., 2007). Their results showed that the treponemal DNA could be isolated from the bone, but only during the acute secondary stage (von Hunnius et al., 2007). Further reasons to explain the low preservation of treponemal DNA included the lack of histone molecules in bacterial genomes, which otherwise protect the DNA from degradation, and the absence of a cell wall, which negatively impacts the preservation of treponemal DNA outside of the host (Mays et al., 2001; von Hunnius et al., 2007). These same factors explain the high preservation of *M. tuberculosis* DNA, which has a protective mycobacterial capsule and a high pathogen load during bone turnover (Bouwman and Brown, 2005). Therefore, a careful consideration of a pathogen's disease sequence should be undertaken before attempting destructive DNA analysis on archaeological remains.

Contamination is another major concern for the use of DNA analysis in paleopathological studies, and researchers need to take steps to reduce contamination and improve their documentation of these steps in publications. Early paleopathological DNA studies did not take steps to avoid potential contaminants due to the belief that contamination was not a concern as the researchers were analysing pathological rather than human DNA (Bouwman and Brown, 2005; Roberts and Ingham, 2008). However, pathological DNA analysis still poses contamination concerns due to the high sensitivity of PCR amplification (Roberts and Ingham, 2008). Recent publications have described many steps which researchers should take to manage the risks of contamination. These steps include ensuring a sterile excavation environment, separating analysis between laboratories, using strict laboratory protocol to reduce the introduction of contaminants, conducting routine monitoring for contaminants, using replicate samples, and critically examining sample results (Roberts and Ingham, 2008). Unfortunately, many researchers either do not use these methods or do not describe them in their publications (Roberts and Ingham, 2008). The following section will describe these steps in more detail, and the benefits which using these steps have for paleopathological research.

The use of a sterile excavation environment is a recent addition to traditional contamination controls. Only 8% of ancient DNA published journal articles examined described using sterile excavation methods, and 90% of the examined papers had no discussion of excavation methods (Roberts and Ingham, 2008). This control method consists of incorporating the prospective DNA analysis into the excavation method and taking extra steps to reduce contamination from the burial environment and post-excavation procedures (Roberts and Ingham, 2008). The use of sterile gloves during excavation is recommended to reduce modern human DNA contamination, and post-excavation treatments with glues or other contaminating preservatives should be avoided (Roberts and Ingham, 2008). Contamination may occur through sample handling and the burial environment so planning excavation strategies which limit these contamination risks will increase the quality of DNA results (Wayne et al., 1999).

Designated work areas for DNA analysis is another factor which can greatly decrease contamination risks. Contamination concerns are of the highest importance during laboratory extraction and amplification, due to the high sensitivity of PCR techniques to contaminants, and special care must be taken to perform these analyses in a designated space (O'Rourke et al., 2000; Roberts and Ingham, 2008). The laboratory space should be separated from that used for modern DNA analysis and areas where the targeted DNA has been previously extracted (O'Rourke et al., 2000; Roberts and Ingham, 2008). Including a description of these procedures in publications such as stating that the laboratories "have had no history of research on the disease-causing organism" (Wayne et al., 1999, p. 468) provides credibility of the results. Other recommendations include using facilities which do not regularly house DNA and separating ancient and modern DNA analyses (Roberts and Ingham 2008). Replication of results in independent laboratories is also recommended to allow for the calculation of interlaboratory error and to confirm unique findings (Roberts and Ingham 2008). Designated workspaces will decrease the levels of contamination during crucial stages of DNA analysis.

Maintaining strict laboratory protocols, which prevent and monitor the introduction of modern DNA, should be carried

out and referenced in paleopathological DNA publications. A database which includes the DNA sequences for all workers should be created to confirm that contamination from workers' DNA did not impact the results (O'Rourke et al., 2000; Roberts and Ingham, 2008). To prevent worker DNA contamination, double gloves, sleeves, masks, and coats should be worn at all times (O'Rourke et al., 2000; Roberts and Ingham, 2008). All protective equipment should either be disposed of after analysis is completed or frequently washed (Roberts and Ingham, 2008). Air filtration will further decrease the potential of laboratory contamination, as well as removing surface contaminants from samples using chemical washes, UV radiation, or physical abrasion as appropriate (Wayne et al., 1999). Many of these laboratory procedures have become standardized, but are not described in 65% of paleopathological publications (Roberts and Ingham, 2008). Strict laboratory standards should be included in publications to ensure high-quality standards of DNA contamination control.

The use of control samples allows researchers to identify sources of contamination and evaluate the authenticity of their results (O'Rourke et al., 2000; Roberts and Ingham, 2008; Wayne et al., 2009). These controls should be used routinely to monitor contamination. The documentation of control use in publications reflects its importance with 89% of papers describing the use of negative controls during DNA amplification (Roberts and Ingham, 2008). However, the number of negative controls is often not recorded, and this is an area of improvement because a single control has a low capability of picking up contaminants (Roberts and Ingham, 2008). A variety of controls provide different sources of information, such as open and closed controls, which are used to differentiate between contaminants caused by PCR setup and reagents (O'Rourke et al. 2000). Some researchers suggest that soil from the excavation site could be used as a control because contaminants are believed to come from the soil of the burial environment (Mays et al., 2001; Roberts and Ingham, 2008). Controls in DNA research should be used more frequently to monitor and evaluate levels of contamination.

To further evaluate the quality of the DNA analysis, samples should be replicated to confirm the initial results (O'Rourke et al., 2000). These replications may include extracting additional samples from different skeletal elements and could be conducted weeks or months apart (O'Rourke et al., 2000; Roberts and Ingham, 2008). Sample NA026 was found to have different spoligotyping signatures when two separate extracts were analysed (Mays et al., 2001). Differential preservation of DR fragments within the bone was proposed to account for these differences (Mays et al., 2001). DNA sequences should also be critically examined to determine if they make phylogenetic sense, with attention given to the product size and the reproducibility of mtDNA results (Roberts and Ingham, 2008). An inverse relationship between fragment size and PCR efficiency should be observed in robust samples (O'Rourke et al., 2000). By keeping these considerations in mind and performing replications of samples, authors can identify contamination when present and confirm the authenticity of the DNA results.

Paleopathological DNA studies are further supported by additional lines of evidence. Using a variety of sources to support an argument is a strength in any publication, and comparing the DNA results with associated remains, using multiple PCR techniques and contextualizing the findings will support the analysis. Associated archaeofaunal remains can be analysed to determine and support the survival of DNA at the site when negative results are observed (Roberts and Ingham, 2008). Using a variety of PCR techniques will support the results of the individual techniques and may provide additional information that would not be observable using just one technique (Mays et al., 2001). Firmly contextualizing the DNA analysis within the sample's historic, ethnographic, and geographic area will provide a deeper understanding of the results and may support a differential diagnosis (Hershkovitz et al., 1998). Therefore, the use of multiple lines of evidence will enhance future palaeopathological research.

5. Conclusion

The use of DNA analysis in paleopathology is crucial for the continued development of this field of research. Despite the challenges which ethics, DNA preservation, and contamination pose on pathological DNA research, the variety of information available from these studies is astounding. Progress can easily be made to improve paleopathological DNA publications, and will strengthen the conclusions drawn about disease origins and human interactions.

6. Literature Cited

Anastasiou E., & Mitchell PD. (2013). Palaeopathology and genes: Investigating the genetics of infectious diseases in excavated human skeletal remains and mummies from past populations. *Gene*, 528, 33-40.

Bouwman AS., & Brown TA. (2005). The limits of biomolecular palaeopathology: ancient DNA cannot be used to study venereal syphilis. *Journal of Archaeological Science*, 32, 703-713.

Donoghue HD., Marcsik A., Matheson C., Vernon K., Nuorala E., Molto JE., Greenblatt CL., & Spigelman M. (2005). Co-Infection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in Human Archaeological Samples: A Possible Explanation for the Historical Decline of Leprosy. *Royal Society*, 272, 389-394.

Gilbert MTP., Djurhuus D., Melchior L., Lynnerup N., Worobey M., Wilson AS., Andreasen C., & Dissing J. (2007). mtDNA From Hair and Nail Clarifies the Genetic Relationship of the 15th Century Qilakitsoq Inuit Mummies.

American Journal of Physical Anthropology, 133, 847-853.

Guhl F., Jaramillo C., Vallejo GA., Yockteng R., Cardenas-Arroyo F., Fornaciari G., Arriaza B., & Aufderheide AC. (1999). Isolation of *Trypanosoma cruzi* DNA in 4,000-Year-Old Mummified Human Tissue From Northern Chile. *American Journal of Physical Anthropology*, 108, 401-407.

Haak W., Brandt G., de Jong HN., Meyer C., Ganslmeier R., Heyd V., Hawkesworth C., Pike AWG., Meller H., & Alt KW. (2008). Ancient DNA, Strontium isotopes, and osteological analyses shed light on social and kinship organization of the Later Stone Age. *PNAS*, 105, 18226-18231.

Hagelberg E., & Sykes B. (1989). Ancient bone DNA amplified. *Nature*, 342, 485.

Harkins KM., & Stone AC. (2015). Ancient pathogen genomics: insights into timing and adaptation. *Journal of Human Evolution*, 79, 137-149.

Hershkovitz I., Donoghue HD., Minnikin DE., Besra GS., Lee OY-C., Gernaey AM., Galili E., Eshed V., Greenblatt CL., Lemma E., Bar-Gal GK., & Spigelman M. (2008). Detection and Molecular Characterization of 9000-Year-Old *Mycobacterium tuberculosis* from a Neolithic Settlement in the Eastern Mediterranean. *PLoS One*, 3, e3426.

Kamerbeek J., Schouls L., Kolk A., van Agterveld M., van Soolingen D., Kuijper S., Bunschoten A., Molhuizen H., Shaw R., Goyal M., & van Embden J. (1997). Simultaneous Detection and Strain Differentiation of *Mycobacterium tuberculosis* for Diagnosis and Epidemiology. *Journal of Clinical Microbiology*, 35, 907-914.

Kaufmann IM., & Ruhli FJ. (2010). Without 'informed consent'? Ethics and ancient mummy research. *Journal of Medical Ethics*, 36, 608-613.

Kirchheimer WF., & Storks EE. (1971). Attempts to establish the armadillo (Dasypus novemcinctus Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. *International Journal of Leprosy*, 39(3), 693-702.

Likovský J., Urbanová M., Hájek M., Černý V., & Čech P. (2006). Two cases of leprosy from Žatec (Bohemia), dated to the turn of the 12th century and confirmed by DNA analysis for *Mycobacterium leprae*. *Journal of Archaeological Science*, 33, 1276-1283.

Mays S., Taylor GM., Legge AJ., Young DB., & Roner-Walker G. (2001). Paleopathological and Biomolecular Study of Tuberculosis in a Medieval Skeletal Collection From England. *American Journal of Physical Anthropology*, 114, 298-311.

Mays S., Fysh E., & Taylor GM. (2002). Investigation of the Link Between Visceral Surface Rib Lesions and Tuberculosis in a Medieval Skeletal Series From England Using Ancient DNA. *American Journal of Physical Anthropology*, 119, 27-36.

Mays S. (2010). Human Osteoarchaeology in the UK 2001-2007: A Bibliometric Perspective. International Journal of Osteoarchaeology, 20, 192-204. Monot M., Honoré N., Garnier T., Araoz R., Coppée J-Y., Lacroix C., Sow S., Spencer JS., Truman RW., Williams DL., Gelber R., Virmond M., Flageul B., Cho S-N., Ji B., Paniz-Mondolfi A., Convit J., Young S., Fine PE., Rasolofo V., Brennan PJ., & Cole ST. (2005). On the Origin of Leprosy. *Science*, 308, 1040-1042.

O'Rourke D., Geoggrey M., & Carlyle SW. (2000). Ancient DNA Studies in Physical Anthropology. Annual Review of Anthropology, 29, 217-242.

Persing DH., Telford III SR., Rys PN., Dodge DE., White TJ., Malawista SE., & Spielman A. (1990). Detection of Borrelia burgdorgeri DNA in Museum Specimens of Ixodes dammini Ticks. *Science*, 249, 1420-1423.

Rafi A., Spiegelman M., Stanford J., Lemma E., Donoghue H., & Zias J. (1994). DNA of *Mycobacterium leprae* Detected by PCR in Ancient Bone. *International Journal of Osteoarchaeology*, 4, 287-290.

Roberts C., & Ingham S. (2008). Using Ancient DNA Analysis in Palaeopathology: A Critical Analysis of Published Papers, with Recommendations for Future Work. *International Journal of Osteoarchaeology*, 18, 600-613.

Saiki RK., Gelfand DH., Stoffel S., Scharf SJ., Higuchi R., Horn GT., Mullis KB., & Erlich HA. (1988). Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science, 239, 487-491. Shendure J., & Ji H. (2008). Next-generation DNA sequencing. *Nature Biotechnology*, 26, 1135-1145.

Smalla K., Cresswell N., Mendonca-Hagler LC., Wolters A., & van Elsas JD. 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *Journal of Applied Bacteriology*, 74, 78-85.

Spigelman M., & Lemma E. (1993). The Use of the Polymerase Chain Reaction (PCR) to detect *Mycobacterium tuberculosis* in Ancient Skeletons. *International Journal of Osteoarchaeology*, 3, 137-143.

Tan SC., & Yiap BC. (2009). DNA, RNA, and Protein Extraction: The Past and The Present. Journal of Biomedical Biotechnology, 2009, 1-10. von Hunnius TE., Yang D., Eng B., Waye JS., & Saunders SR. (2007). Digging deeper into the limits of ancient DNA research on syphilis. *Journal of Archaeological Science*, 34, 2091-2100.

Wayne RK., Leonard JA., & Cooper A. (1999). Full of Sound and Fury: The Recent History of Ancient DNA. *Annual Review of Ecological Systems*, 30, 457-477.

Zink AR., Sola C., Reischl U., Grabner W., Rastogi N., Wolf H., & Nerlich AG. (2003). Characterization of *Mycobacterium tuberculosis* Complex DNAs from Egyptian Mummies by Spoligotyping. *Journal of Clinical Microbiology*, 41, 359-367.

Zink AR., Sola C., Reischl U., Grabner W., Rastogi N., Wolf H., & Nerlich AG. (2004). Molecular Identification and Characterization of *Mycobacterium tuberculosis* Complex in Ancient Egyptian Mummies. *International Journal of Osteoarchaeology*, 14, 404-413.