Investigation of the Causative Agent of Canine Leproid Granuloma Infection Cases in the Northeastern United States

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Investigation of the Causative Agent of Canine Leproid Granuloma Infection Cases in the Northeastern United States

Nicole Marcotte

UNH Honors Animal Science

Honors Capstone
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Abstract

Canine leproid granuloma syndrome (CLGS) is a form of disease in dogs of many breeds that is characterized by a skin infection in the subcutis and/or dermis. The granulomas that form as a result of infection are typically non pruritic, alopecic, raised, and firm. These mass-like granulomas can ulcerate, causing further infection beneath the skin surface, and are present in areas of the body such as the ears, head, neck, and extremities. CLGS has been known to infect dogs of different breeds and ages, but has most commonly been reported to infect shorthaired, large sized breeds. This disease is suspected to be caused by a nontuberculous form of Mycobacterium spp and has displayed significant sequence identity with mycobacterial species such as Mycobacterium tilburgii, Mycobacterium interjectum, M. simiae and Mycobacterium genavense. The etiological agent species is currently unknown. Because of this, scientists and veterinarians alike are eager to discover the species of bacterium causing infection in cases of CLGS. Using a variety of methodologies including DNA extraction, polymerase chain reactions (PCR), gel electrophoresis, and gene sequencing, the etiological agent was identified as a mycobacterial species. DNA was obtained and amplified from the available tissue samples from previous cases of CLGS infection and sent off for sequencing to determine the species of Mycobacterium spp. Findings supported the genus of Mycobacterium spp, as the gross lesions, histopathological diagnosis, and molecular diagnostic tests produced results consistent with mycobacterial infections. Understanding the species of the etiological agent causing disease can further aid veterinarians in prescribing the correct treatment for animals and can further help scientists learn more about the unknown bacterium, how it spreads in the environment, and how to prevent the spread of further infection.
Introduction

Problem Description

In veterinary medicine, it is common to see disease present in canines as well as other animals stemming from a bacterial infection of some sort. Several strains of *Mycobacterium* *spp* have been identified to cause disease ranging from subclinical to clinical in canines of various breeds. *Mycobacterium* *spp* can affect both humans and animals, reinforcing a zoonotic component of disease depending on the type of bacteria causing infection, mode of transmission, and other factors. Mycobacterial infections can be either tuberculuous (MTb), causing either tuberculosis or leprosy, or nontuberculuous (NTM), causing a range of illnesses that can affect systemic body parts such as the lungs, skin, and blood.\(^1\,^2\) Research focused on different strains and species of *Mycobacterium* *spp* involves studying the tissue microscopically, cytologically, and histopathologically to understand more at the immune level. By using polymerase chain reactions (PCR) to amplify the DNA of this bacterial species, scientists can analyze target segments of the genome and compare them to other known species of *Mycobacterium* *spp*. This can provide medical professionals and scientists alike with the ability to establish the most plausible treatment methods for the specific type of *Mycobacterium* *spp* at hand and further help determine the bacterium’s pathogenicity and virulence.

Canine leproid granuloma syndrome, or CLGS, is an illness showing prevalence within the past 50 years caused by an unknown species of *Mycobacterium* *spp*. CLGS first presented itself in canines in the year 1973 in Zimbabwe in Boxers and Bull Mastiffs.\(^3\) Since then, numerous cases of CLGS have been reported worldwide, with prevalence in countries such as Africa, Australia, New Zealand, Brazil, and areas of the United States.\(^4\,^5\) From the analysis of
several diagnostic testing methods using skin biopsies from affected animals, scientists were able to discover the genus of the bacterium as *Mycobacterium spp*, but not the species.\textsuperscript{1,4,6,7} Culturing of this specific bacterium in laboratory settings has been rather unsuccessful as the etiological agent is suspected to be saprophytic, therefore other methods of analysis must be performed to deduce the species.\textsuperscript{1,6} Because of this, it is important that scientists determine the primary bacterium involved with CLGS infection cases at the species level by utilizing methods of DNA extraction and sequencing, as well as other methods described previously, to offer a reliable and effective treatment method for affected patients. Additionally, discovery of the etiological agent can offer scientists and medical professionals alike the opportunity to understand more about this new bacterium itself. Identification of the etiological agent of CLGS is also essential for reasons pertaining to the bacterium’s epidemiology, as certain *Mycobacterium spp* are zoonotic.

**Available Knowledge**

Canine leproid granuloma syndrome, or CLGS, is an infectious form of dermatitis characterized by dermal or subcutaneous growths on the pinnae, areas of the head, and in lesser cases the extremities.\textsuperscript{1,4} No involvement of systemic organs or lymph nodes are commonly reported with CLGS.\textsuperscript{1,8} This syndrome is caused by an unknown bacterial species of the genus *Mycobacterium spp*, suspected to be nontuberculous.\textsuperscript{1} These growths appear as single or several nodules, papules, or plaques on or under the skin that are often painless or cause little pain, are nonpruritic, and cause alopecia.\textsuperscript{1,4,9} These growths can ulcerate and predispose the dog to develop secondary bacterial infections.\textsuperscript{1,4,9,10} Certain canine breeds are predisposed, such as short hair coat breeds and larger sized breeds, but all ages can be affected by CLGS.\textsuperscript{4,7}
Histopathologically, canine leproid granulomas consist of nodular masses of predominantly macrophages with fewer neutrophils. Macrophages contain variable numbers of small, non-staining linear bacteria, and bacteria can also be found extracellularly.\textsuperscript{6,8–10} Lymphocytes, plasma cells, and giant cells in varying numbers are also present, with mixed numbers of neutrophils seen as well.\textsuperscript{6,8} Acid-fast histopathologic stains, such as Ziehl-Neelson and Fite-Faraco methods, can be used to highlight the bacteria in the lesions.\textsuperscript{6,8,9} 10\% buffered formalin fixed, paraffin embedded (FFPE) tissues biopsied from infectious cases of CLGS are utilized for various diagnostic procedures.\textsuperscript{1,8} When DiffQuik staining is utilized, bacteria are presented as “negative images,” where the background is stained with the Romanovksy reagents but the bacteria itself are not stained due to the lipid-rich cell wall.\textsuperscript{6} In an acid-fast stain using Ziehl-Neelson carbol-fuchsin solution, acid alcohol, and methylene blue solution from a fine needle aspirate of tissue, bacteria stain a positive bright red with a blue background.\textsuperscript{6,8} In this stain, neutrophils can be intact or degenerate, with macrophages containing non-stained rod-shaped structures inside, consistent with mycobacterial infections.\textsuperscript{6,8} Lesions can either be pyogranulomatous or granulomatous in patients with CLGS. In pyogranulomatous growths, neutrophils, multinucleated giant cells, and epithelioid macrophages rush to the site of infection in large numbers and act as the first line of defense.\textsuperscript{1} In granulomatous lesions, mostly multinucleated giant cells and epithelioid macrophages are present at the site of infection, often with helper T cell lymphocytes.\textsuperscript{6,8,11} Additionally, CLGS granulomas can form when the immune cells described previously are recruited to the site of infection and cluster together, forming a mass.\textsuperscript{6,8}
In previous cases of CLGS, scientists and researchers alike have recognized numerous species of *Mycobacterium* *spp* as disease-causing agents. These species include *Mycobacterium simiae* and *Mycobacterium interjectum*, two species of nontuberculous *Mycobacteria* (NTM).¹,⁷,⁸,¹¹ The bacterium *M. simiae* was suspected due to the slow-growing, low pathogenicity nature of the bacterium, while *M. interjectum* was suspected due to its close relation to the disease-causing agent as well as its presence in the *M. simiae* clade.¹,⁷,⁸ The disease-causing agent of CLGS has also been identified to share high nucleotide sequence homology with other NTMs such as *M. tilburgii* and *M. genavense*.⁷ In one specific case mentioned, the agent of CLGS identified shared a sequence identity of over 99% over 510bp for *M. tilburgii*, with shared sequence identity to *M. simiae*, *M. interjectum*, and *M. genavense* reaching 98.8% over 519bp.⁷ One article even discovered a tuberculous *Mycobacterium* (MTb) present in a case of CLGS, naming it *M. murphy* for lack of a previously documented species name.¹ Two articles determined that the disease-causing agent of CLGS shared 99.4% nucleotide identity over 519bp with a currently existing mycobacterial strain, IWGMT-90413, determined from PCR analysis of identical sequences over a 350bp region, as well as PCR analysis of the 16S rRNA gene.¹,⁷

Because this syndrome is caused by a species of *Mycobacterium* *spp*, it is important to understand how this organism functions, modes of transmission, and how it appears in different diagnostic testing methods. Instances of mycobacteriosis can be present within numerous animal types including mammals, reptiles, fish, humans, and various wildlife species. *Mycobacterium spp* are a species of bacteria known for years to cause infections in both humans and animals with symptoms ranging from mild to severe. Most *Mycobacterium spp* are saprophytic, acquiring food and therefore energy by absorbing broken-down organic material, and most are fastidious.¹,⁶
Because of the mycobacterial species involved in CLGS possessing intricate nutrient requirements for growth, isolation in culture media has been challenging and rather unsuccessful, presenting a complication when studying this syndrome. These bacteria contain lipid-rich cell walls with a mycolyl-arabinogalactan-peptidoglycan (mAGP) complex that is characterized by mycolic acid, a compound vital for survival, growth, membrane permeability, and virulence of the bacterium. Mycobacterium spp falls into two categories, nontuberculous and tuberculous, both known to be disease-causing. Nontuberculous infections are typically not contagious, however an infection caused by a tuberculous bacterium can be spread from animal to animal.

Several nontuberculous Mycobacteria (NTM) are known to cause mycobacteriosis, including previously mentioned species such as M. simiae, M. interjectum, M. tilburgii and M. genavense. NTM are typically aerosolized and transmitted via ingestion, inhalation, and through penetration by a foreign body. Following inhalation of an NTM or a MTb, the bacterium is phagocytized by macrophages in the lungs. NTM are often located environmentally in dust, municipal and natural water, and soil, with the capacity to produce biofilms. NTM infections present symptoms affecting areas such as the lungs, skin and soft tissue, lymph nodes, and blood. Symptoms are often vague, including fever, weight loss, decreased appetite, and more, with certain symptoms depending on the site of infection. In most cases of CLGS infection, the analysis of biopsied tissue from affected patients supported the discovery of NTM bacteria. Treatment for NTMs typically involve antibiotics, including either the use of one type or more than one combined for the desired effect. These antibiotics include rifampicin, clarithromycin, and azithromycin. M. simiae has been documented to cause pulmonary disease and can predispose cardiovascular disease, diabetes, and cancer as a result. Some nonspecific symptoms
experienced may include a productive cough, hemoptysis, dyspnea, fever, night sweats, malaise, and weight loss.\textsuperscript{11} \textit{M. interjectum} has been previously reported to cause lymphadenitis in children, as well as pulmonary disease in adults.\textsuperscript{15} Nonspecific symptoms of this infection vary but are typically similar to those of \textit{M. simiae}.\textsuperscript{15} The documentation of symptoms and related disease, either as a secondary form or from predisposition, for infections with \textit{M. tilburgii} and \textit{M. genavense} show significant similarities to that of \textit{M. simiae} and \textit{M. interjectum} and therefore the detailed description for these aspects of disease is omitted.

Alternatively, tuberculous mycobacteria (MTb) infections causing mycobacteriosis include \textit{M. tuberculosis}, \textit{M. leprae}, both being classified as significant diseases for humans as well as animals, and \textit{M. bovis}, all zoonotic diseases. MTb are typically transmitted through inhalation of aerosol droplets from infected individuals.\textsuperscript{16} To treat all MTb infections, antibiotics are typically used, either alone or in combination with others. \textit{M. tuberculosis} can be fatal if not treated properly, however not all individuals infected become ill. This bacterium attacks regions of the body such as the kidney, spine, and brain. \textit{M. tuberculosis} bacteria invade the lungs and can settle there as well as move through the blood to other bodily regions, causing greater infection. Animals such as primates and guinea pigs face the highest risk but can pose risks to dogs and cats as well. \textit{M. leprae} is typically not fatal but can leave permanent damage if not treated properly or in a timely fashion. This bacterium attacks the skin, mucous membranes, and peripheral nerves of the body, leaving external lesions such as discolored patches of skin, painless ulcers on feet, and loss of eyebrows or eyelashes. Because internal nerves are affected as well, muscle weakness or paralysis of the hands and feet, enlarged nerves, and sight issues may result. Animals facing the highest risk for disease caused by \textit{M. leprae} include humans,
mangabey monkeys, and armadillos. *M. bovis* is similar to *M. tuberculosis* in that it does not cause all affected individuals to display signs of sickness. This bacterium attacks the lungs, lymph nodes, and other parts of the body, causing symptoms depending on where the bacteria affect. *M. bovis* is commonly found in unpasteurized dairy products and affects humans, cattle, elk, bison, and deer. Despite the repeated examination of tissue and presence of NTM in skin samples from patients diagnosed with CLGS, it is important to still consider MTbs as infectious agents, as a case of this syndrome characterized by infection with an MTb has been recorded.¹

For the purposes of this project, several laboratory techniques were essential to achieving the desired results of species identification. Tissue samples from available cases in the Northeastern US were biopsied, fixed in formalin, and routinely processed for histopathology. preserved in the laboratory using paraffin wax sliced into thin sections, as well as fixing the samples in formalin. The preservation of tissue was essential to protecting its integrity for each scientific experiment and to prevent decay of the material being studied in the laboratory.¹⁷ Tissue samples from the available cases were analyzed via histopathologic diagnosis to identify the bacteria present microscopically. This allowed for the general identification of *Mycobacterium spp*, and further specified which mycobacterial species to consider as a etiological agent.⁹ DNA was extracted from the FFPE tissue samples in efforts to prepare the samples for gene amplification. The amount of extracted DNA was quantified using a fluorometer and the TapeStation apparatus to measure if there was a sufficient amount left to conduct further species identification testing. Polymerase chain reactions (PCR) was done for 16S V3-V4, rpoB, and hsp65 genes to amplify extracted DNA fragments from the tissue samples.¹⁸ These genes were targeted specifically, as 16S V3-V4 supports the detection of
general bacteria, whereas hsp65 and rpoB support the presence of *Mycobacterium spp* in the samples. Using the amplified DNA, gel electrophoresis was conducted to separate the fragments based on their size and charge. The totality of the steps described above provided results for the sequencing of amplified DNA in efforts to speciate the *Mycobacterium spp* present in the tissue samples. Once the genes were sequenced, they were placed into a genetic sequence database to identify the species of bacteria present in each sample.

**Rationale**

By taking 10% formalin fixed, paraffin embedded biopsy samples from affected patients with CLGS, various diagnostic testing methods can be used to help identify the unknown species of pathogenic bacterium. The identification of this *Mycobacterium spp* species is necessary to help establish proper treatment for patients affected with CLGS. However, despite described success achieved with the use of single antibiotics or a combination of them, identification of the pathogenic agent would allow medical professionals to better assess the duration of medication usage as well as how to reduce the spread of disease and prevent infection. Additionally, if the bacterium continues to thrive in the environment and cause disease in animals globally, further studies must be conducted to ensure it does not have zoonotic potential or the potential to mutate and cause more significant disease than what is currently described in patients with CLGS.

**Global Aim**

Canine leproid granuloma syndrome has been described in canines of different breeds, sizes, and ages across the globe. Because of this, it is important to identify the species causing disease to help scientists and medical professionals understand ways to limit the spread of disease on both the small and large scale. By examining the hosts and environments globally that
allow CLGS to thrive, the agent of disease can be better understood, reducing the amount of animals that will require treatment if diagnosed with CLGS.

Specific Aim

Canine leproid granuloma syndrome has become more prevalent in several areas of the Northeastern United States. Because this bacteria is suspected to be spread through environmental contaminants, issues may arise in the Northeastern US specifically as much of the natural flora and environment are preserved in terms of canopy cover and water supply. Therefore, it is essential for scientists to characterize the causative agent in order to prevent a nationwide spread of infection among animals in the Northeastern US.
**Materials and Methods**

CLGS was diagnosed by histopathology, and the formalin-fixed, paraffin-embedded (FFPE) tissues were used for this study. DNA was extracted from the tissue samples using QIAGEN FFPE Kit according to the manufacturer’s procedure. The instructions from the manufacturer are described in detail in Appendix A. Extracted DNA was quantified using an Invitrogen fluorometer and the Agilent TapeStation 4200 apparatus, both according to the manufacturer’s procedure. The instructions from the manufacturer for both the Invitrogen fluorometer and the Agilent TapeStation 4200 apparatus are located in detail in Appendix B. Polymerase chain reactions (PCR) were done for 16S, rpoB, and hsp65 genes, followed by gel electrophoresis of amplified DNA. The PCR trials run on gel electrophoresis conducted in this experiment are detailed in Appendix C. A temperature gradient methodology was run for the rpoB and hsp65 genes, and can be found in further detail in Appendix D. The DNA amplified from PCR was sequenced and compared to sequences present in the genetic sequence database.
Results

Demographic Data of Available Cases

Table 1: Below is a table describing the case information for each of the 6 affected patients, received by both the New Hampshire Veterinary Diagnostic Lab (NHVDL) at University of New Hampshire as well as the Animal Health Diagnostic Center (AHDC) at Cornell University. The first six cases described are canines, and the remainder of cases within the table are included as controls for this study.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Age (yrs)</th>
<th>Breed</th>
<th>Sex</th>
<th>Lesion Location</th>
<th>Clinic Location</th>
<th>Case Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Basset Hound</td>
<td>MN</td>
<td>Multifocal (head, neck, thigh)</td>
<td>Ipswitch, MA</td>
<td>NHVDL</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>Shepherd Mix</td>
<td>MN</td>
<td>Left pinna</td>
<td>Laconia, NH</td>
<td>NHVDL</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Boxer</td>
<td>FS</td>
<td>Left hind leg</td>
<td>Hopkinton, NH</td>
<td>NHVDL</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Terrier Mix</td>
<td>FS</td>
<td>Bilateral pinna</td>
<td>Pembroke, MA</td>
<td>NHVDL</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>Labrador Retriever</td>
<td>MN</td>
<td>Left pinna</td>
<td>Churchville, NY</td>
<td>AHDC</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>Greyhound</td>
<td>FS</td>
<td>Left pinna</td>
<td>Winsted, CT</td>
<td>AHDC</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>Domestic Shorthair</td>
<td>FS</td>
<td>Cranial right mammary</td>
<td>Ashfield, MA</td>
<td>NHVDL</td>
</tr>
<tr>
<td>2</td>
<td>~2</td>
<td>Domestic Chicken (unsure of breed)</td>
<td>F</td>
<td>Visceral</td>
<td>Pembroke, MA</td>
<td>NHVDL</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>Thoroughbred Horse</td>
<td>MN</td>
<td>Left side, behind girth</td>
<td>Stewartstown, PA</td>
<td>AHDC</td>
</tr>
</tbody>
</table>

Gross Lesions

The typical lesions described by submitting veterinarians were one or more dermal or subcutaneous masses present on either one or both ears, the head, the neck, or an extremity. Punch or wedge biopsies displayed reddish-pink, non pruritic, raised, irregular, hairless masses ranging in size and shape. In some of the documented cases, the masses were scabbed and
presented signs of infection underneath the skin where ulceration occurred. Throughout the cases available, the ears were most commonly affected, where the lesions remained localized and did not spread.

Figure 1: The figure above displays a gross canine leproid granuloma lesion observed from a 10 year old, spayed female greyhound in Winsted, CT. The skin of the left pinna is expanded by a pink, raised, hairless nodular mass.

*Histopathologic Diagnosis*

The general histopathologic diagnosis described in each of the CLGS cases supports mycobacterial infection through cell staining and morphology. The histopathology of the samples revealed multifocal, modular to diffuse pyogranulomatous patterns of inflammation, consistent
with general lepromid granulomas. Moderate numbers of acid-fast bacilli were observed within macrophages and observed extracellularly in necrotic tissue.

Figure 2: The figure above displays macrophages throughout the lesions containing variably abundant acid-fast bacilli under 400x magnification using a Ziehl-Neelsen acid fast stain.
Figure 3: The figure above displays the results of the hematoxylin and eosin stain in two different magnifications. In A, a photomicrograph of a canine leproid granuloma biopsy sample is visualized, where the dermis and subcutis are infiltrated by coalescing nodular aggregates of macrophages and neutrophils under 20x magnification. In the inset B, the lesions are characterized by sheets of pyogranulomatous inflammation under 200x magnification.

Molecular Diagnostic Results

Figure 4: The figure above shows the quantity of intact DNA obtained from the TapeStation apparatus after extraction from FFPE tissue samples. Notice that the DNA is not degraded and
retains high molecular weight after the extraction process. Further quantifiable data for each sample is located in Appendix B.

Figure 5: The figure above depicts the results of the second round of PCR testing utilizing 16S, Cytochrome B, 18S, and 16S V3-V4 primers visualized on 2% agarose gel for each sample. The intensity of the band indicates the amount of DNA product resulting from PCR testing, where brighter bands indicate higher product yield.
Figure 6: The figure above shows the results of the third round of PCR testing utilizing 16S V3-V4, hsp65, and rpoB primers visualized on 2% agarose gel for each sample. The intensity of the band indicates the amount of DNA product resulting from PCR testing, where brighter bands indicate higher product yield.
Figure 7: The figure to the left depicts the annealing temperature gradient range of 45°C-60°C for the rpoB primer on the left, with the annealing temperature gradient range of 45°C-55°C for the hsp65 primer on the right. Black arrows on the left image of rpoB indicate the primer-specific-amplified DNA band whose size is determined from the DNA ladder on the right.
Discussion

After the examination of all results obtained from this experiment, it is deduced that they fit the context of what is seen in scientific literature regarding canine leproid granulomas and their subsequent infection-causing syndrome. When looking at the demographic data, it is clear that the gross lesions, histopathologic diagnosis, and molecular diagnostic results all compare to those obtained in similar case studies in different areas of the nation, as well as globally. In terms of the project goals, they were achieved, as DNA was successfully extracted from 5-micron-thick sections of FFPE tissue samples from cases of canine leproid granulomas diagnosed by histopathology. Additionally, the DNA extracted was successfully amplified. Sequencing of the DNA extracts is pending, which will allow for speciation of the *Mycobacteria* spp.

FFPE tissues were used because they are typically all that remains from biopsy samples long term. Most labs retain the FFPE tissue blocks indefinitely, so it is an easily accessible bank of tissue samples to use for retrospective studies similar to this one. Because preservation of tissue in this fashion can be rather damaging and cause fragmentation within the DNA of the sample, quantification via the Invitrogen fluorometer and the Agilent TapeStation 4200 apparatus was necessary to ensure substantial DNA was retained from the samples after extraction to be used for gene amplification. Likewise, utilizing the TapeStation apparatus was beneficial in determining if the DNA recovered from extraction retained its integrity and molecular weight. PCR in efforts to amplify the DNA from each sample was essential to generate multiple copies of the target genes for sequencing. Amplifying the target genes, rather than the entire genome, conserved time and resources, as well as aided in determining if the genes of interest that help distinguish the species of mycobacteria were located in any of the available case samples.
Specific genes of 16S V3-V4, rpoB, and hsp65 were used as the target genes in this experiment because these were target genes described in previous scientific literature and obtained results for *Mycobacterium* species identification. In the second round of PCR, target genes of 16S, cytochrome B, and 18S were utilized as positive control sets. The general 16S gene targets the presence of general bacteria, where the cytochrome B gene picks up mitochondrial DNA among many species, and the 18S gene is specific to eukaryotes. Gel electrophoresis was necessary for separating the fragments of DNA based on their size and charge, and helped identify the base pair length of each fragment in efforts to see if the amplified gene is present in the tissue samples. Utilizing water in the DNA ladders during gel electrophoresis functioned as a negative control to ensure no contamination was present in the tissue samples being studied. Because the PCR was successful in amplifying genes for the gel electrophoresis trials, bands were expressed in specified regions throughout the gel for the target genes. It is important to note that the methods of PCR provided quantifiable results that supported the inclusion of *Mycobacterium* in the samples utilized, meaning the DNA was not too small or degraded to use and the suspected genus organism was confirmed as the etiological agent. It is also important to note that the first round of PCR was unsuccessful and yielded no results for the gel electrophoresis trial, providing rationale for the annealing temperature gradients done for the rpoB and hsp65 genes. Causes of failure for the first PCR trial may be attributed to the dilution of case DNA in the samples run or due to runoff from the FFPE tissue samples inhibiting PCR. The annealing temperature gradients for rpoB and hsp65 were completed to determine the optimal temperature for the primers to aid in recombining the DNA into its double stranded form. The methods utilized for the purpose of this experiment were proven successful in terms of extracting and amplifying DNA, as well as preparation for sequencing to determine the identification of the mycobacterial species.
Limitations

Few limitations arose throughout the duration of this project, with some notable instances including the method of DNA extraction, PCR turnaround time and temperature, and contamination in the controls used for gel electrophoresis. Extracting DNA from FFPE tissue has shown potential to degrade DNA and fragment it smaller or larger than desired. Using various methods of quantifiable measures, it was determined that the amount and integrity was retained for the most part. However, utilizing DNA that was preserved using a different method may have yielded higher results in terms of how much DNA was amplified and able to be used in gel electrophoresis. Because the process of PCR was unsuccessful in the first trial, two limitations arose: one pertaining to the time it took for the PCR to be completed, and another for the annealing temperature at which this process was completed. The process of PCR takes slightly over an hour, and if the process of PCR was shorter, more trials could have potentially been run to achieve desired results. Additionally, the annealing temperature could have also caused a limitation on the first trial run, as running the PCR at the correct annealing temperature may have prevented an incorrect run and saved DNA to be used for a PCR trial at a correct temperature. Lastly, contaminants in the water for the negative controls of gel electrophoresis were present in the first trial and showed dye staining in the control ladders of the gel. Contaminated controls could potentially lead to contamination in other samples, as water is used to create the master mix utilized in each well, affecting each sample studied. Despite the few limitations faced throughout the totality of this project, the desired results were still obtained in an efficient and reliable fashion that could be replicated in similar laboratory settings.
Conclusion

In conclusion, the methodologies used to extract, quantify, and amplify DNA from available tissue samples were proven successful. The detection of bands in the PCR product for 16S V3-V4 supports the presence of general bacteria among the samples, with the detection of rpoB and hsp65 supporting the presence of *Mycobacterium* in the samples tested. In terms of next steps, the sequencing of amplified DNA from the 16S V3-V4, rpoB, and hsp65 gene sections will provide a genetic sequence to be run through a genetic sequencing database. Once run through the database, this information will allow the speciation of the *Mycobacterium* present among the canine leproid granuloma infection cases analyzed through various methods.

**Specific Aim Relevance**

The findings of this project are substantially relevant to the specific aim. Because of the prevalence of CLGS nationwide as well as worldwide, obtaining results to be utilized for sequencing was essential to confirming that a bacterial species was the etiological agent, as well as confirming its presence as a *Mycobacterium* species. Additionally, examining the results from the gross lesions, histopathologic diagnosis, and the molecular diagnostics allowed the determination of localized effects of the etiological agent on animals included in the study.
References:


https://tvmdl.tamu.edu/2019/03/20/leproid-granuloma-in-a-boxer-dog/


https://www.cdc.gov/nontuberculous-mycobacteria/about/index.html


Appendix

Appendix A

The following protocol was used for the extraction of DNA from FFPE tissue samples using the QIAGEN FFPE kit:

Using a scalpel, extra paraffin was trimmed off each sample block to help rid the sample of excessive paraffin. Eight sections of 5-10μL thickness were cut from the sample block, with the first 2-3 sections discarded if the sample surface had been exposed to air. Immediately, each section was placed in a microcentrifuge tube (2mL) and Citrisolv (1mL) was added to each tube to aid in dissolving the paraffin attached to each section. Each tube was vortexed vigorously for 10 seconds to ensure correct mixing for dissolvement and then centrifuged at a speed of 20,600 RPM for 2 minutes at room temperature to separate the paraffin from the sample. Citrisolv was removed via pipetting using a Gilman P-1000, leaving the remaining sample pellet at the bottom of each tube, using fine pipetting tips with a Gilman P-200 to remove any residual Citrisolv. In each tube, 96% ethanol (1mL) was added to each sample pellet and vortexed vigorously for 5 seconds, allowing the ethanol to extract the residual Citrisolv. The samples were centrifuged at a speed of 20,600 RPM for 2 minutes at room temperature to further separate the paraffin from the sample. The ethanol was removed via pipetting using a Gilman P-1000, leaving the remaining sample pellet at the bottom of each tube, using fine pipetting tips with a Gilman P-200 to remove any residual ethanol. Each tube was opened and incubated at room temperature for 10 minutes, allowing time for the evaporation of all residual ethanol. The sample pellets were resuspended in a mixture of Buffer ATL (180μL) and proteinase K (20μL) and vortexed vigorously for 5 seconds in efforts to digest nucleases that degrade DNA and RNA in the samples and isolate the
necessary genomic DNA. The samples were incubated at 56°C for 1 hour to ensure lysis of the sample and incubated for 1 hour at 90°C to partially reverse the formaldehyde modification of nucleic acids. The tubes were briefly centrifuged to remove drops of sample lodged inside the lid after incubation. Buffer AL (200μL) was added to the sample, vortexed vigorously, and 96% ethanol (200 μL) was added and vortexed vigorously to produce a homogenous solution. Each tube was briefly centrifuged to remove drops of sample inside the lid. The entire lysate, consisting of protein, nucleic acids, buffer, and ethanol, was transferred to the QIAamp MinElute column and centrifuged at 8000 RPM for 1 minute. The column was then placed in a clean collection tube (2mL), discarding the collection tube containing the flow through. Buffer AW2 (500μL) was added to the column and centrifuged at 8000 RPM for 1 minute. The column was then placed in a clean collection tube (2mL), discarding the collection tube containing the flow through. The column was centrifuged at a speed of 14,000 RPM for 3 minutes to dry the membrane and placed into a clean microcentrifuge tube (1.5mL), discarding the collection tubes containing the flow through. Buffer ATE (55μL) was administered to the center of each tube’s membrane to elute the DNA from the sample. The lid was closed and incubated at room temperature for 5 minutes to increase DNA yield, then centrifuged at a speed of 14,000 RPM for 1 minute.

Appendix B

The following protocol was used for the quantification of extracted DNA using an Invitrogen Fluorometer in efforts to calculate the stock concentration of each sample:

Generic buffer (2189μL) was combined with fluorescent reagent (11μL) to create a master mix. Aliquots of the master mix (200μL) were added to 9 different tubes, allowing each
to consist of buffer (199μL) and reagent (1μL). Each unique sample (1μL) was added to its corresponding tube and briefly vortexed. The calculated stock concentrations for each of the samples was obtained [1: 72.2ng/μL, 2: 43.0ng/μL, 3: 52.4ng/μL, 4: 36.8ng/μL, 5: 6.96ng/μL, 6: 1.25ng/μL, 7: 2.22ng/μL, 8: 36.6ng/μL, 9: 38.6ng/μL].

The following protocol was used for the quantification of extracted DNA using the Agilent TapeStation 4200 apparatus in efforts to calculate the quantity of DNA that can be used for PCR:

Loading dye (9μL) and undiluted sample (1μL) were loaded into strip tubes and vortexed moderately to ensure full sample mixing. The contents were placed into the Agilent TapeStation 4200 apparatus and ran for 14 minutes [1-9: ≥ 10000 bp].

Appendix C

The following protocol was used for the first trial of PCR with inclusion in gel electrophoresis on the available tissue samples:

A master mix of the KAPA enzyme and subsequent nucleotides (72μL), 16S V3-V4 forward primer (8.4μL), 16S V3-V4 reverse primer (8.4μL) and water (31.2μL) were combined into a single test tube. This step was completed twice more using the forward and reverse primers for the hsp65 and rpoB genes. Each DNA sample from the nine cases used was diluted to 2ng/μL for PCR. The master mix (10μL) was combined with sample DNA (2μL) for each of the nine samples tested, and the totality of this mix was placed into thermocycler plates. Two additional samples were utilized as controls, one from dog DNA and another from shark DNA. The PCR was set to run in the thermocycler for one hour, with annealing temperatures for 16S V3-V4 and hsp65 set at 55°C and annealing temperatures for rpoB set at 60°C. Both plates were
run for 30 cycles. After PCR, the samples were loaded into their respective wells in a 48 well apparatus containing 2% agarose gel and run using gel electrophoresis [no results obtained].

The following protocol was used for the second trial of PCR with inclusion in gel electrophoresis on the available tissue samples:

A master mix of the KAPA enzyme and subsequent nucleotides (84μL), 16S forward primer (9.8μL), 16S reverse primer (9.8μL) and water (36.4μL) were combined into a single test tube. This step was completed again using the forward and reverse primers for the 16S V3-V4, cytochrome B, and 18S genes. Each DNA sample from the nine cases used was undiluted for PCR. The master mix (10μL) was combined with sample DNA (2μL) for each of the nine samples tested, and the totality of this mix was placed into thermocycler plates. Two additional samples were utilized as controls, one from dog DNA and another from shark DNA. The PCR for plate 1 including 16S and cytochrome B was set to run in the thermocycler for one hour and 27 minutes with an annealing temperature of 50ºC. The PCR for plate 2 including 18S and 16S V3-V4 was set to run in the thermocycler for one hour and 36 minutes with an annealing temperature of 57ºC for 18S and 55ºC for 16S V3-V4. Both plates were run for 35 cycles. After PCR, the samples were loaded into their respective wells in a 48 well apparatus containing 2% agarose gel and run using gel electrophoresis [bands observed in Figure 5].

The following protocol was used for the second trial of PCR with inclusion in gel electrophoresis on the available tissue samples:

A master mix of the KAPA enzyme and subsequent nucleotides (84μL), 16S V3-V4 forward primer (9.8μL), 16S V3-V4 reverse primer (9.8μL) and water (36.4μL) were combined into a single test tube. This step was completed again using the forward and reverse primers for
the rpoB and hsp65 genes. Each DNA sample from the nine cases used was undiluted for PCR. The master mix (10μL) was combined with sample DNA (2μL) for each of the nine samples tested, and the totality of this mix was placed into thermocycler plates. Two additional samples were utilized as controls, one from dog DNA and another from shark DNA. The PCR was set to run in the thermocycler for one hour, with annealing temperatures for 16S V3-V4 and hsp65 set at 55ºC and annealing temperatures for rpoB set at 60ºC. After PCR, the samples were loaded into their respective wells in a 48 well apparatus containing 2% agarose gel and run using gel electrophoresis [bands observed in Figure 6].

**Appendix D**

The following protocol was used to determine the range of annealing temperatures for the rpoB and hsp65 genes under PCR conditions:

A master mix of the KAPA enzyme and subsequent nucleotides (57.6μL), hsp65 forward primer (67.2μL), hsp65 reverse primer (67.2μL) and water (297.6μL) were combined into a single test tube. This step was completed again using the forward and reverse primers for the rpoB gene. Each DNA sample from the nine cases used was undiluted for this PCR. The master mix (125μL) was combined with sample DNA (10.5μL) for each of the nine samples tested, and an increment of this mix was placed into thermocycler plates. Two additional samples were utilized as controls, one from dog DNA and another from shark DNA. The PCR for the rpoB gene was set from a range of 45ºC-55ºC and ran for 1 hour and 42 minutes, whereas the PCR for the hsp65 gene was set from a range of 45ºC-60ºC and ran for 1 hour and 42 minutes. After PCR, the samples were loaded into their respective wells in a PCR annealing temperature apparatus containing 2% agarose gel and run using gel electrophoresis [bands observed in Figure 7].