Characterization of *Corynebacterium pseudotuberculosis* biovar *equi* isolates obtained from Cal Poly horses and review of application of transposons to better prevent pigeon fever in horses

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Chapter 1

Literature Review: *Corynebacterium pseudotuberculosis* and Transposon Mutagenesis Methods for Determination of the Pathogen's Virulence

Corynebacterium pseudotuberculosis

Pigeon fever is an infectious disease in the horse caused by the bacteria *Corynebacterium pseudotuberculosis*; biovar *equi* is the strain that infects mainly horses. The organism lives in soil and survives best in drought conditions. The bacteria are spread primarily by flies and enter wounds or open flesh where they spread to the local lymph nodes (Lenz, 2013). The most common sign of the disease is external abscessing resembling a pigeon's breast in the pectoral region; pain and lameness may or may not accompany it. Abscesses can also develop on the ventral abdomen, the mammary gland, sheath, or legs (Lenz, 2013). Fewer horses develop more serious forms of the disease like internal abscesses that interfere with internal organs or ulcerative lymphangitis, a condition where the infection causes inflammation, swelling, and lesions on limb tissues (Fannin, 2012). Photographs of pigeon fever conditions can be viewed in Appendix I. There is a high incidence of pigeon fever in Texas, California, and the Midwest, and currently there is no vaccine against the bacteria.

Microbiologically, *C. pseudotuberculosis* is a Gram-positive, facultative intracellular (can grow inside or outside cells) pathogen that possesses pleomorphic forms such as coccoid to filamentous rods measuring 0.5-0.6 micrometers in width and 1.0-3.0 micrometers in length (Bastos et al., 2012). The bacterium is non-capsulated, non-sporulating, and does not have flagella; however, it does have fimbriae (Bastos et al., 2012). *Corynebacterium pseudotuberculosis* is a mesophilic facultative anaerobe. Dorella et al. (2006) reported that it cultured well at 37° Celsius with a pH between 7.0-7.2. In a later study, Bastos et al. (2012) found that it grew well across a wider pH range from 7.0-8.0. Enriched media, such as brain heart infusion (BHI) agar or broth and blood agar, are best to grow the bacteria (Bastos et al., 2012). The bacterial colonies on agar are opaque, cream to yellowish in color, and concentrically ringed. Bacterial growth in broth creates a biofilm on the surface that forms precipitates when agitated.

Biochemically, *C. pseudotuberculosis* produces the enzymes catalase, phospholipase D, and urease and ferments carbohydrates such as maltose, mannose, glucose, and less frequently, galactose (Bastos et al., 2012). It does not ferment lactose, produce gas, or have proteolytic activity; it is also oxidase negative and beta-hemolytic (performs complete hemolysis) (Bastos et al., 2012). With regards to nitrate reduction, this specific strain reduces nitrate to nitrite and is sensitive to the antibiotic streptomycin.

The leading virulence factor of *C. pseudotuberculosis* is phospholipase D (PLD), an exotoxin produced by the bacterium. PLD hydrolyzes ester bonds in sphingeomyelin from mammalian cell membranes, causing them to become abnormally permeable which leads to the spread of the bacteria from the initial site of infection to the rest of the host's body (Dorella et al., 2006). In addition, PLD triggers dermonecrosis, which contributes to the distribution of *C. pseudotuberculosis* from the skin to blood vessels and eventually to lymphatic vessels, causing severe edema where the abscesses enlarge and mature (Bastos et al., 2012).

Although a clinically affected horse can be usually detected by physical examination, blood work, and ultrasound imaging, a great deal of the biology of *C. pseudotuberculosis*

remains unknown. Past investigators have mapped the bacterium's genome, like in Ramos et al.'s 2012 project that sequenced the Cp316 strain, biovar *equi*, of the bacteria from a Californian horse, but no studies have compared genomes, or proteomics, of isolates from different regions, including different regions in the same state. Such a study would reveal whether the organism expresses virulence factors (chiefly phospholipase D) to variable degrees, whether expression of such proteins changes over the course of an outbreak, or whether some strains lack certain virulence factors altogether.

For this senior project, we investigated the genome of the organisms in isolates derived from horses with pigeon fever at Cal Poly and around the state. The sequenced genome will then be used to develop a transposons library to facilitate an understanding of *C. pseudotuberculosis*' virulence and pathogenicity mechanisms.

Transposon Mutagenesis Methods for Determination of the Pathogen's Virulence

Ramos et al. proposed that combining mate-paired libraries, which is sequencing involving creation of ligated random fragments representing the whole sample, with Ion Torrent PGM sequencing increases efficiency at reading and assembling the *C. pseudotuberculosis* biovar *equi* genome anew. Ion Torrent PGM is a benchtop high-throughput sequencer, but it is also the "first post-light sequencer." It identifies bases by detecting the release of hydrogen ions during DNA synthesis (Ramos et al., 2013).

After developing the genome assembly of the bacteria using Ion Torrent PGM and mate-paired library, the researchers compared the resulting genome with that of the *equi* strain previously added at NCBI. This was obtained by another platform that also used a mate-paired library. The results were astounding. There were only 16 gaps in the Ion Torrent Mate-pair individual mapping, indicating great coverage of the entirety of the genome (Ramos et al., 2013). Regarding new genes, their de novo assembly for *C. pseudotuberculosis* added an additional 91 kb of sequence to the reference genome displayed at NCBI (Ramos et al., 2013)! Furthermore, with a little more research and information from software, results showed that some of the missing regions in the reference genome actually represent a Corynephage and nitrate-reductase operon (Ramos et al., 2013). A total of 248 coding sequences in the *C. pseudotuberculosis* genome represented the new regions that were assembled with post-light sequencing (Ramos et al., 2013).

The extensive coverage of the bacterial genome by the Ion Torrent Mate-pair allowed for the discovery of new sequences that were not previously recorded in the reference genome. Because of this, the library's efficiency is definitely high, especially with the advantages that come with using mate-paired libraries in the study's semiconductor platform, such as low cost, reduced need for computational resources and optical readout, and improved data usage. The results from Ramos et al.'s work could be applied to further studies requiring the bacteria's genome, like the development of a transposons library for *C. pseudotuberculosis* to identify DNA sequences that encode proteins of interest, possibly relating to pathogenicity. A study in 2006 by Dorella et al. already set out in that direction, but with the biovar *ovis* strain. In general, Dorella et al. used transposons in insertional mutagenesis to identify genes encoding exported proteins.

Transposons, also known as transposable elements (TEs) or jumping genes, are genetic sequences that can move from one location on the genome to another. There are two classes of TEs. Class 2 TEs move by means of a "cut and paste" mechanism using the enzyme transposase,

which is required for their excision and insertion, and their ends consist of terminal inverted repeats that assist in recognition by transposase (Pray, 2008). Class 1 TEs, also known as retrotransposons, move by replicative mechanisms; they produce RNA transcripts and then depend on reverse transcriptase to reverse transcribe the RNA sequences back into DNA which is inserted into the target location (Pray, 2008). Both class 1 and 2 TEs have flanking direct repeats which play a role in the insertion of the TE (Pray, 2008).

When inserting into the genome, transposons can interrupt the normal DNA sequence, creating gene mutations with a variety of effects. For example, nearby genes could be turned off, preventing their ability to create protein, or the genes could be turned on, increasing the amount of protein made. In bacteria, transposons often carry genes that are responsible for resistance to antibiotics, helping the bacteria survive (van Opijnen, 2013).

In Dorella et al.'s experiment, the *in vivo* insertional mutagenesis consisted of constructing a library of the bacterial strains each containing a single transposon. The researchers used the TnFuZ transposition system which combines a version of the Tn*4001* TE with the DNA fragment encoding the mature *Enterococcus faecalis* alkaline phosphatase gene (*phoZ*) (Dorella et al., 2006). High levels of *phoZ* can induce bone disease, hyperthyroidism, and damage to the liver. After the *C. pseudotuberculosis* cells were transformed with the nonreplicative TnFuZ-containing plasmid, insertional mutants were isolated on brain heart infusion agar. One thousand five hundred mutant *C. pseudotuberculosis* colonies were attained, and 34 of those had positive *phoZ* activity (Dorella et al., 2006). This transposon, then, played a role in silencing the *phoZ* gene.

After the DNA of the 34 *phoZ* mutants were extracted and sequenced, analysis of the nucleotides showed that the flanking repeats were similar to 21 *C. pseudotuberculosis* loci that code for membrane proteins like fimbrial and transport subunits (Dorella et al., 2006). This discovery is especially exciting because fimbriae are used to help anchor the pathogen onto the host surface, allowing for colonization and eventually infection. These genes then are potential, promising targets for the creation of a new vaccine! A future study could incorporate the method of *in vivo* insertional mutagenesis in the *C. pseudotuberculosis* biovar *equi* strain and hopefully find genes to target for a possible vaccine for pigeon fever.

As described, genomic sequencing and transposon mutagenesis are very useful in linking a phenotype to a genotype. Such studies have led to the identification of thousands of virulence genes in pathogens, not including *C. pseudotuberculosis* (van Opijnen et al., 2013). According to van Opijnen et al. (2013), transposons are the most frequently used tool for genome-wide genotype-phenotype studies. Van Opijnen et al. discuss four main transposon sequencing techniques used to analyze microorganisms. The basis of the four methods involves the following: purification of the genomic DNA from the mutants, cleavage of the DNA, attachment of one or more adaptors (DNA used to link ends of two other DNA molecules/proteins) to the DNA fragments to facilitate polymerase chain reaction (PCR) amplification of the fragments containing the transposon, and massively parallel sequencing (MPS; also known as second-generation sequencing) of the fragments to define the location of the transposon and the relative amount of mutants with a transposon at that site (van Opijnen et al., 2013).

The first technique described is the high-throughput insertion tracking by deep sequencing (HITS). After shearing of the DNA, the ends are repaired, a poly(A) tail is added, and adaptors are ligated. The PCR step is next, and the resulting products undergo size selection on a gel and purification before being sequenced. With the HITS method, virulence genes were identified (van Opijnen et al., 2013). A similar technique called transposon-directed insertion site

sequencing (TraDIS) was able to identify genes essential for growth in a rich medium (van Opijnen et al., 2013). In TraDIS, the process is the same as HITS' until after the PCR step; PCR products are sequenced directly instead of being selected and purified first (van Opijnen et al., 2013). As a tradeoff for lengthy sample preparation protocols, both of these methods can use any transposon.

The other two methods, insertion sequencing (INSeq) and transposon sequencing (Tn-seq), are nearly identical to each other. They can be applied to determine genes essential for growth in rich medium, the fitness effect, and genetic interactions (aggravating and alleviating) between query genes and the rest of the genome (van Opijnen et al., 2009; van Opijnen et al., 2013). These techniques both utilize the Himar 1 *Mariner* transposon; a single nucleotide change in the terminal inverted repeats of this transposon introduced recognition sites for *Mme*I, a type IIS restriction endonuclease that makes a 2bp staggered cut 20 bases downstream of the recognition site (van Opijnen et al., 2013). Fragments consisting of the left and right transposon ends are made, and the 16bp of flanking DNA that is also produced is enough to find the location of the transposon insertion in the bacterial genome (van Opijnen et al., 2013).

Because a Himar I *Mariner* transposon could be used to create a library of insertional mutants from *C. pseudotuberculosis* isolates, application of Tn-seq is ideal. By using MPS, Tn-seq is a highly sensitive, robust, and reproducible method to explore quantitative genetic interactions in the bacteria (van Opijnen et al., 2009). Additionally, Tn-seq can be easily applied to different environments, such as various growth media or infection models; therefore, further Tn-seq studies working with *C. pseudotuberculosis* biovar *equi in vitro* is a possible next step. This could reveal phenotypes that will help in identifying new gene functions, determining virulence mechanisms, and advancing our knowledge of the pathogen's metabolic requirements.

Chapter 2

Senior Project Background and Objectives

For my senior project, I was involved with a pigeon fever research study titled, "Determination of strain and comparison of genome sequence and transposons in *Corynebacterium pseudotuberculosis* biovar *equi* isolates obtained from horses from different counties in California." With the sequenced genome, we are hoping to develop a transposons library that will help us find genes associated with encoding the bacterium's virulent and pathogenic factors. This two-year project was proposed in Fall quarter, 2013, and it kicked off at the start of Winter quarter, 2014. Dr. Sandra Jouglard from the Biology Department and Dr. Kim Sprayberry from the Animal Science Department were the primary investigators, and two other students and I were the original student staff. Three more students were later added to form a team that would have members continuing the project after some of us graduated.

My team needed to collect samples from our research subjects (horses) and use our skills and techniques acquired from related class labs to carefully grow the bacteria and run biochemical tests to positively identify the strain. Once identified, results from the bacterium's DNA extraction and genomic sequencing will provide us with genomic characterization of the bacterium that we can 1) compare with strains from other regions, and 2) analyze to begin the formation of a transposons library. The library will offer information regarding *C. pseudotuberculosis*' pathogenicity and virulence which we can then use to better treat effected animals.

During the two quarters that we have worked on the project with limited resources, we positively identified a few samples of *C. pseudotuberculosis* and accomplished DNA extraction of one of those samples. We also found a company where we will send our genomic extractions to be sequenced.

Chapter 3

Results

From the first round of Gram staining, a few of the many samples that we were growing in BHI broth tubes stained purple, indicative of a Gram-positive bacteria, and showed the normal bacteria's rod formation. The Gram stain of one of those samples, labeled HA8 standing for name of the horse the sample was retrieved and round of collection, is pictured in Figure 1. Some of the Gram-positive stains showed coccus-shaped bacteria, and a few stains had both cocci and rods; however, this was not alarming since *C. pseudotuberculosis* is pleomorphic, or able to change its shape and size due to environmental conditions.

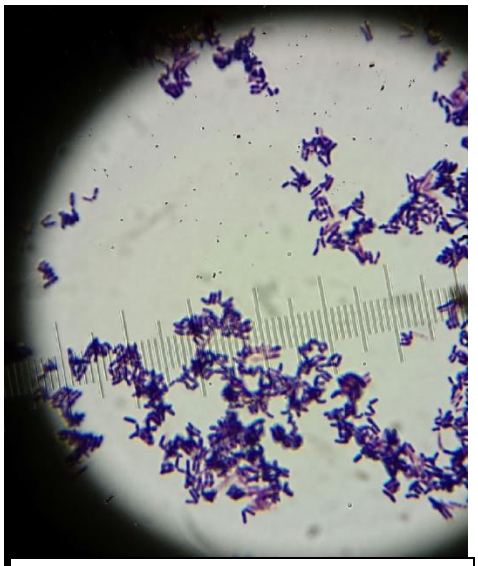


Figure 1. Gram stain results of sample HA8 at 1000X total under immersion oil: purple rods, showing a likely identification of *C. pseudotuberculosis*.

After the Gram stains, denitrification and ammonification tests were performed on three questionable samples to confirm identification of *C. pseudotuberculosis*. Since growth was detected in all three tubes, we proceeded to ammonium ion detection. As shown in Figure 2, samples R2A1 and HB1 produced ammonium while R1A1 did not. Denitrification tests followed to check all three samples for nitrate reduction to nitrite, which is characteristic of this strain of bacteria. Our results, also shown in Figure 2, show that one of the samples, R2A1, was able to do so, further confirming the presence of *C. pseudotuberculosis*; the other two were disregarded as probable candidates for eventual bacteria DNA extraction.

From the results of the (sometimes repeated) biochemical tests, we chose and paired up the best candidates for positive identification of *C. pseudotuberculosis*; they were samples HA8 with HA5 and R2A1 with R2A2. The colonies from each of those samples were inoculated into four BHI broth tubes for DNA extraction. So far, the genomic DNA for HA8 has been extracted and is being frozen until shipment to the sequencing company.

Figure 2. Tables of results for Nitrate tests on selected samples

Denitrification	Sample R1A1	Sample R2A1	Sample HB1
Growth	+ (turbid)	+ (turbid)	+ (turbid)
Gas in tube?	-	-	-
Fizzing after addition of Sulfamic acid?	-	+ (prolonged)	-
Nitrate to nitrite reduction?	No	Yes	No

Ammonification	Sample R1A1	Sample R2A1	HB1
Growth	+	+	+
Color after adding Nessler's?	Yellow	Orange-brown/slight darkness	Orange-brown/slight darkness
Ammonium ion detected?	No	Yes	Yes

Chapter 4

What I Learned by Doing

Cal Poly's "learn by doing" motto has been heavily emphasized throughout the course of my research experience. In the period of time I had to work on the project, I was able to learn and accomplish so much. First off, my team was essentially finding funding, lab space and materials while we were starting up the lab work for the research project. Since we started from scratch with no funding, making any research progress was daunting and quite hectic, but we managed to make do with what we were able to access. During the first phase of the project, I was in charge of drafting a letter to be sent to foundations, companies and laboratories requesting donations of funds and/or materials. In addition, I assisted with looking for lab space where we could perform our bench work, researching grants to help fund our project, and locating places that were willing to let us borrow or lend us the equipment and materials we needed for the time being.

Improving my skills in reading and *understanding* scientific research articles and literature reviews on *C. pseudotuberculosis* and transposons was a big part of my learning process during the project. I was not aware of the fact that our bacterium of interest is pleomorphic, especially when we witnessed its pleomorphism firsthand in the lab while we were gram staining; however, after reading scientific articles on the bacterium and related species, I figured out that the shape-shifting abilities of *C. pseudotuberculosis* are completely normal. In addition, I learned completely new information while researching information about transposons and their mechanisms. After completing my own literature review, I now understand how transposons can be applied in insertional mutagenesis and used in our project to study the bacteria's virulence. Transposons insert themselves into genes, and development of a library with these transposon mutations can provide us information on the consequences of the mutated gene, such as the prevention of encoding a specific protein that could be a virulence factor.

This project gave me the opportunity to fine-tune my laboratory skills during biochemical testing and other bench activities. The lab skills and methods that I applied included inoculating and growing bacteria, streaking a colony for isolation, purifying and storing bacteria cultures, and performing biochemical tests (i.e., Gram and nitrate) on our samples from two infected Cal Poly horses to confirm that the identities of our samples were indeed *C. pseudotuberculosis*. I also improved my discipline in writing and documenting everything in my laboratory notebook. The information put into my notebook is important as it could provide a reference for protocols and a record for any results or observations.

I had to do multiple Gram stains of the bacteria in both quarters. These re-stains were done to ensure that the bacteria from the stock culture that we were planning to use for DNA extraction retained its original characteristics and possible identity of *C. pseudotuberculosis*. There were a few times when we got contamination of our samples on the media in both plates and tubes. These situations, although unfortunate, drove my team and me to put a stronger emphasis on meticulously practicing sterile techniques. The sterile techniques that became more enforced included washing hands before and after performing lab work, wearing gloves and lab coat, flaming opened ends before and after pouring or inoculating, always handling the samples and sterile material close to the flame, and parafilming tubes and plates for better prevention of contamination.

In addition to being able to practice skills, I learned new technical skills. For the first time and with advice from a kind microbiology technician, I made BHI agar and broth media using instructions I modified from an online source. It was such a new experience for me, and although the process was a little rocky at the start, I ended up preparing sterile sets of BHI agar plates and broth tubes! The protocol I used is listed in Appendix II. Later in the course of the project, I was asked to prepare BHI slants. Since I have made BHI media before, I felt more confident in my ability to carry out this task. The protocol for preparing the BHI slants can be found in Appendix II. Autoclaving is another technique that I learned from preparing the BHI media. I took great care in selecting the right materials that could enter the autoclave and made sure to operate the machine safely.

For the DNA extractions, I assisted in making a solution called "Solution I" that was needed for the process. Since we did not have the exact ingredients for the solution, my teammates and I improvised and we were still able to create the correct solution. The instructions for preparing Solution I are listed in Appendix II. Before starting the DNA extraction of our positively identified bacteria, centrifuging of the samples was necessary to create the cell pellet. As seen in Figures 3 and 4, a small amount of each sample was carefully transferred into centrifuge tubes, which were spun in the centrifuge at 10k rpm for five minutes. The resulting supernatant liquid was diluted with distilled (DI) water and disposed of, leaving the pellet at the bottom. The bacterial cell pellets were then stored in a freezer for at least two hours before being used in the DNA extraction process.

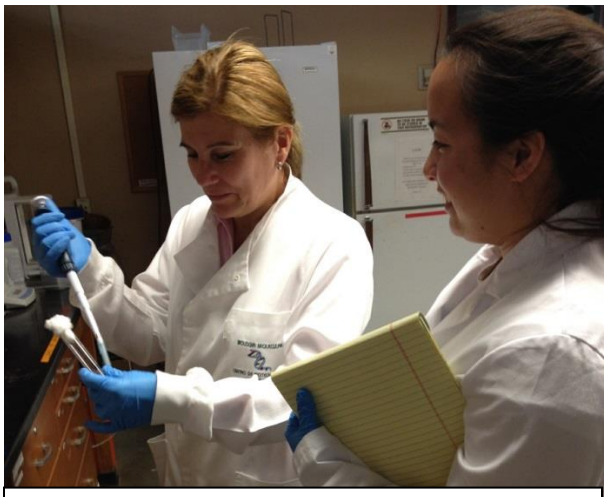


Figure 3: Dr. Jouglard, left, micropipetting a *C. pseudotuberculosis* culture sample into centrifuge tubes as I, right, observe.

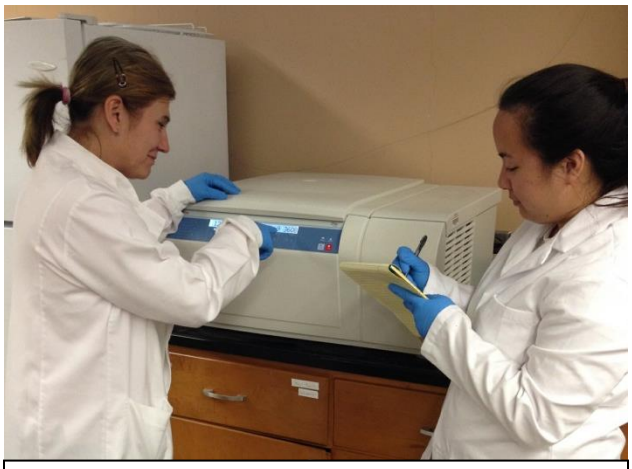


Figure 4: Dr. Jouglard, left, operating the centrifuge to form a pellet of bacterial cells as I, right, record notes.

Chapter 5

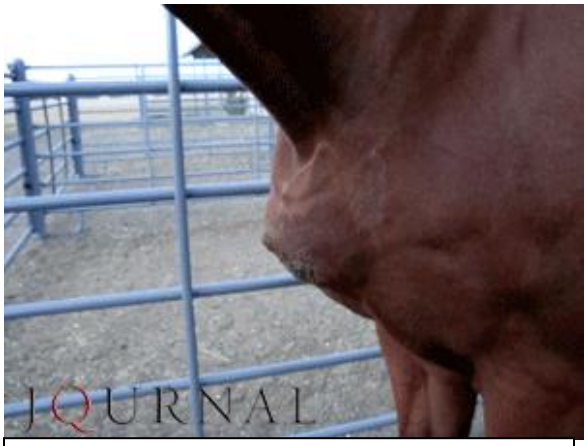
Ideas for Improvement

What could be improved for the next phases of the project are the amount and availability or resources that my team can have access to. We had no funding or designated lab space and very limited materials when we started the research project, so it was difficult to get the work going. My research advisor just recently told us about our first grant that we had received last month, so the money can be used to buy much needed materials; this will greatly relieve the labor of having to hunt down willing people to lend us supplies.

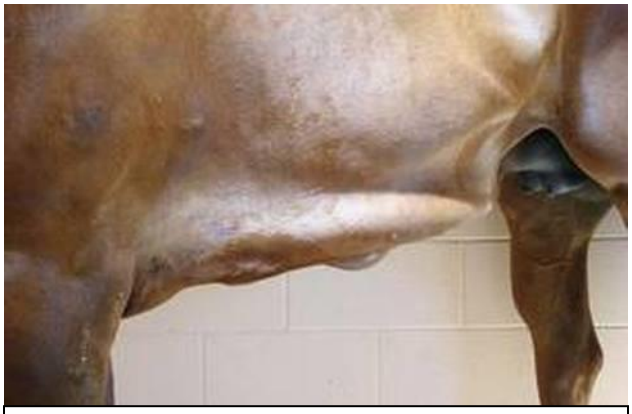
Although some expectations of the project were established, they were not clearly communicated or documented, contributing to a shortcoming of this project. Due to inadequate management and lack of a formal project specification document for team members to reference, our efforts were at times aimless and misguided. In addition, many times when my teammates and I came in to the lab to work, we could not seek help from our research advisor since she either was preoccupied with other duties or did not show up for the scheduled sessions. Project meetings were also delayed or cancelled multiple times without notice, creating frustration among the group.

For future work in this project, responsibilities need to be clearly identified and taken up. In the past two quarters, some of the group members seemed to have their own agenda or were not on the same page as others, making it difficult for everyone to collaborate efficiently. Three things are crucial to facilitate a smoother project: organization and management, better communication, and more instruction from the research advisor during lab work. If these aspects can be improved, we can in turn improve the rate of progress with the research.

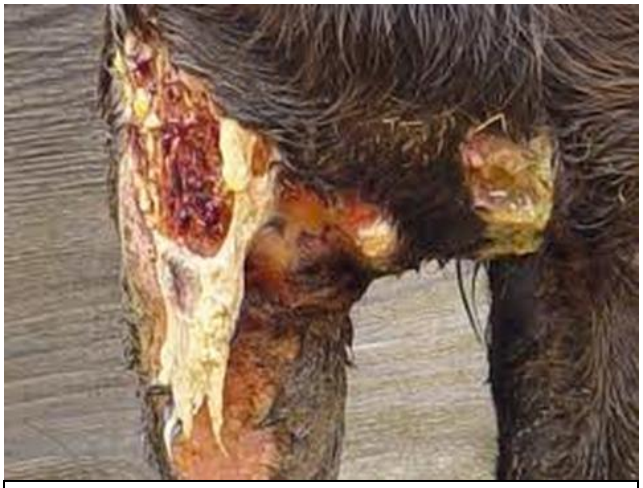
Appendix I: Clinical Signs of Pigeon Fever in Horses



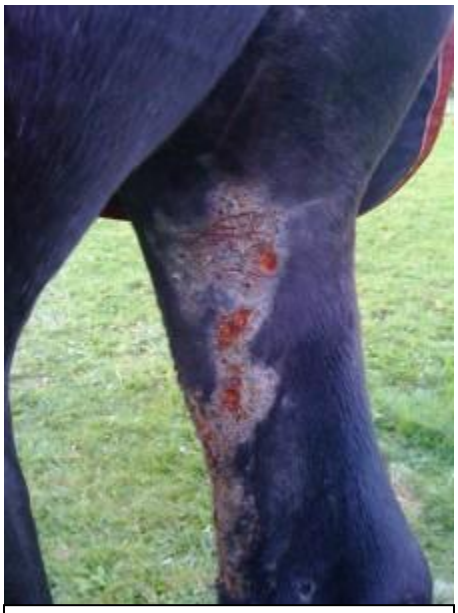
Abscessing in the horse's pectoral region resembles a pigeon's breast, thus the term "pigeon fever" (*Journal* photo)



Abdominal swelling with abscesses reaching maturity (mwveterinaryservices.com photo)



Tissue damage in the pectoral region after rupture of multiple large abscesses (mwveterinaryservices.com photo)



Ulcerative lymphangitis in the limb; swelling and ulcerative lesions are seen (equinemld.com photo)

Appendix II: Lab Protocols

Brain Heart Infusion Broth and Agar Preparation (modified from www.condalab.com)

Materials: Erlenmeyer flask (500mL), distilled (DI) water, hot plate, stir bar, tubes, plates, tube caps, BHI media powder, agar powder (15%), weigh scale, weigh paper, funnel, tube stand, aluminum foil

Broth Procedure:
1. Weighed and transferred 9.25g BHI powder to 500mL Erlenmeyer flask along with a stir bar.
2. Flask was filled with 250mL DI water and stirred vigorously on the hot plate until dissolved.
3. With a funnel, 10mL of BHI broth was poured into each tube.
4. Tubes were capped and autoclaved (followed instructions on the autoclave machine for operation).
5. Cooled tubes were stored in the refrigerator.

Agar Procedure:
1. Weighed and transferred 9.25g BHI powder and 3.75g agar powder to 500mL Erlenmeyer flask along with a stir bar.
2. Flask was filled with 250 mL DI water and mixed well with stirring and boiling on the hot plate until completely dissolved.
3. Flask with media was covered with aluminum foil and autoclaved (followed instructions on the autoclave machine for operation).
4. After being autoclaved, 20mL of BHI media was poured into each plate between 2 Bunsen burners to minimize contamination.
5. Agar plates were allowed to set and cool with lids placed at a slight angle to allow for evaporation and less condensation on the lids; this step was also done by the 2 Bunsen burners to minimize contamination from surrounding air.
6. Set agar plates were covered and stacked on top of each other with lid side down and parafilmed together for storage in refrigerator.

Brain Heart Infusion Slant Preparation (modified from www.condalab.com)

Materials: Eight tubes, tube caps, Erlenmeyer flask (250mL), DI water, hot plate, stir bar, BHI media powder, agar powder (15%), weigh scale, weigh paper, funnel, tube stand

Procedure:
1. Weighed and transferred 1.2g agar powder and 2.96g BHI powder to 250mL Erlenmeyer flask along with stir bar.
2. Flask was filled with 80mL DI water and mixed well with stirring and boiling until completely dissolved.
3. With a funnel, 10mL of BHI broth was poured into each of the eight tubes.
4. Tubes were capped and autoclaved (followed instructions on the autoclave machine for operation).
5. Tubes were cooled in an angled position in the tube racks to allow for a slanted agar surface to form.
6. Slants were stored in the refrigerator.

Preparation of Solution I, a DNA Extraction Reagent (modified from http://www.protocol-online.org)

Materials: Tris-HCl (pH 7; 10mM; 2mL of solution 0.5M), EDTA (pH 8; 10mM; 2mL of solution 0.5M), NaCl (300mM; 6mL of solution 5M), DI water, Falcon tubes, pH meter, HCl (1M), Milli-Q water, beaker (250mL)

Procedure:
1. The pH and molarity of the Tris-HCl that we had (pH 8; 1M) was adjusted to get the correct Tris-HCl properties needed as the ingredient for Solution I. To do this, 3mL of 1M HCl was added to 12mL of 1M pH8 Tris-HCl, and enough mL of Milli-Q water added to the Tris-HCl. The pH level of the adjusted Tris-HCl solution was checked with the pH meter, and the amount of Milli-Q water needed to adjust the molarity was calculated using $C_1V_1=C_2V_2$.
2. In a 250mL beaker, combine 2mL of 0.5M pH 7 Tris-HCl, 2mL of 0.5M pH 8 EDTA, and 6mL of 5M NaCl.
3. Enough DI water was added to make up a total volume of 100mL of Solution I.
4. Solution I was transferred to and stored in a Falcon tube.

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