Eastwood, Raelyn A.
BIOL342 F03

Testing an Unknown Bacterial Isolate Obtained from My Border Collie's Foot Pad Through Physiological Testing, Genome Sequencing, and Antibiotic Resistance

Introduction:

Microbes are found throughout the vast expanse of our Earth and even space. They can even live in very harsh conditions such as extremely hot hydrothermal vents, deep within the ocean, and arctic ice and glaciers. Microbes can grow in aerobic conditions as well as anaerobic conditions and yet some can grow in both. I hypothesize I would isolate a Gram-positive, aerobic bacterium common to the microbial community found on dog's footpads and in between their toes. Quite a bit of research has been done in the veterinary community in regards to skin conditions in canines. However, little research has been done that emphasizes canine pedal microbiomes.

Methods to explore the diversity of skin microbiomes on canines and felines are lacking, and therefore, there is little information into the abundance of bacterial communities present. However, dermatologic microbiomes can potentially describe a patient's susceptibility to diseases and how they may react to infectious and non-infectious diseases. Just as in humans, the skin microbiome also plays an important role in prevention of diseases. *Staphylococcus aureus, Micrococcus ssp., Acinetobacter,* were found to be the primary resident species. *Streptococcus, Bacillus, Clostridium, Proteus mirabilis, E. Coli, and Corynebacterium ssp.* were thought to be transient species because they weren't as abundant on a per patient basis *(*Weese, J. S. 2013). Other skin infections are associated with fungal growth as well.

One common skin disease among canines is known as *Malassezia* dermatitis. *Malassezia* is a fungus genus that is commonly found on skin of a variety of different species, to include humans. Antifungal treatment for *Malassezia* dermatitis can be quite costly. Terbinifine is one such antifungal drug that can be used and works by inhibiting cell wall synthesis in fungus. Terbinifine did not persist on canine skin as it does on humans. This result may be due to the pH range of canine skin, which is between 4.8 and 9.9, whereas human skin pH is typically 5.2 -5.6 (Gimmler, J., et al 2015)

Methods:

Initially, I had selected two different environments to sample from. I chose to sample my pups right, front foot pad and her tooth. After inoculating a TSA agar plate with the selected environmental samples, three days elapsed that provided enough time for the bacteria to grow on the media. A yellow bacterial colony from her foot pad was selected from the agar plate and cultured. Over several weeks, several new TSA agar plates were inoculated to purify the culture using aseptic techniques to prevent contamination (Lab 2).

Gram-staining techniques were carried out to determine whether the isolated species was Gram-positive or Gram-negative, and whether the sample was contaminated.

DNA was extracted using a PowerSoil DNA Isolation Kit (Lab 5) and the genome was sequenced in the sequencing lab. Genome information was uploaded into BaseSpace Illumina web software (Lab 5) in order to use bioinformatics analyses to identify the bacterial isolate.

The bacterial isolate was subjected to various physiological tests including a catalase test,

an oxidase test, a fluid thioglycollate test, and an API20E test strip. The catalase test is important in determining whether the bacterial isolate contains catalase which catalyzes the release of oxygen from hydrogen peroxide. The oxidase test distinguishes Gram-negative enteric bacteria from Gram-positive pseudomonad bacterial species by determining whether the bacteria contain cytochrome-c oxidase. The thioglycollate test works as a visualization to reveal the oxygen class of the bacteria. Depending on the location of the bacterial growth within the sterile broth, determines the oxygen class. Aerotolerant bacteria grow throughout the sterile broth equally. Facultative bacteria grow throughout the sterile broth, but are more concentrated towards the top where they are exposed to more oxygen. Strict anaerobes prefer to grow at the bottom of the tube where there no oxygen, and strict aerobes prefer to grow at the top of the media, closest to abundant oxygen. Finally, the API20E test strip tests for certain metabolic traits such as and that ultimately end in a number combination that can be searched for in a database to the corresponding species associated with the numbers (Lab 6).

Finally, antibiotic susceptibility of the bacterial isolate will be conducted utilizing the Kirby-Bauer Method which uses a disc diffusion test. If the bacteria tend to avoid growing near the discs, then the results indicate that they are susceptible to that type of antibiotic (Lab 9).

Results:

Physiological Testing:

Gram staining of the unknown bacterial isolate revealed a Gram-negative species, (Figure 1, 2). The fluid thioglycollate test revealed a facultative anaerobic species that is able to persist with or without the presence of oxygen. The oxidase test was positive, indicating my unknown bacterial species contained cytochrome c oxidase. The catalase test was also positive in which the unknown bacteria reacted with H2O2, indicating presence of catalase enzyme.

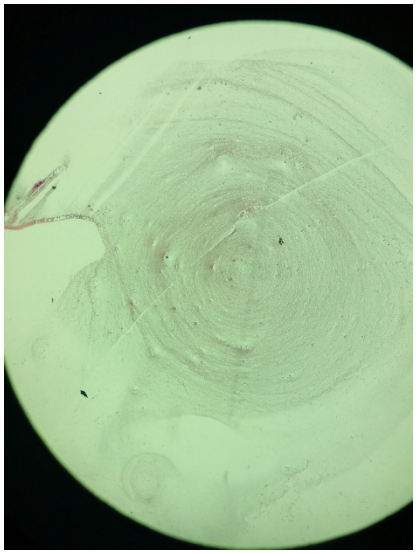

Figure 1: Gram negative stain 10x

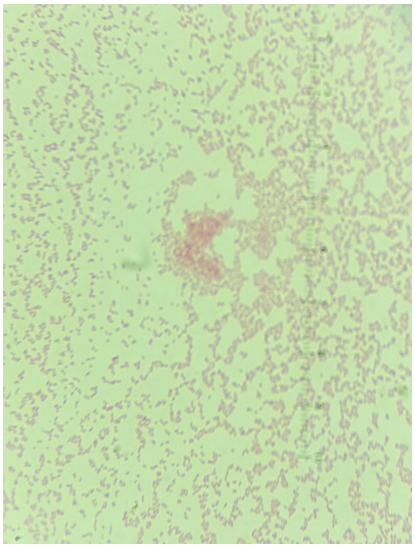

Figure 2: Gram negative stain 100x

Physiological testing of the API20E strip, Figure 3, revealed very little information for my unknown bacterial species. Two physiological API20E tests were conducted to determine possible physiological traits. The first API20E test was inconclusive as I had failed to add

enough bacteria to my sample medium. The test strip had also dried out over the weekend. The second API20E test revealed positive results for VP, GEL, and GLU tests. The MAN, INO, SOR, RHA, SAC, MEL, AMY, and ARA tests were all negative with a slight yellow coloring around the cupule. The remainder of the tests were negative.

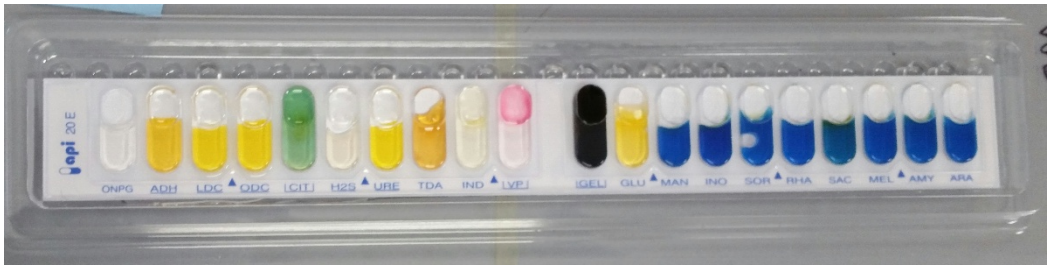

Figure 3: API20e Test Strip #2 indicating positive results in VP, GEL, and GLU after the addition of secondary reagents in TDA, IND, VP, and GLU.

Genome Sequencing Analysis:

DNA extraction, genome sequencing, and Bioinformatics analysis provided more accurate taxonomic results than the API20E test kit, Figure 4. According to Kraken Metagenomics, my isolate was 99.93% identified down to species level as *Proteus mirabilis*. 22% of the sequence was identified as *Proteus* strain HI4320, while 30% was identified as *Proteus* strain BB2000. Both of these strains are associated with urinary tract infections (Schaffer, J. et al. 2016). Only 6% of the DNA extracted was unable to be sequenced, and the remaining 42% identified as *Proteus mirabilis*, without a strain type.

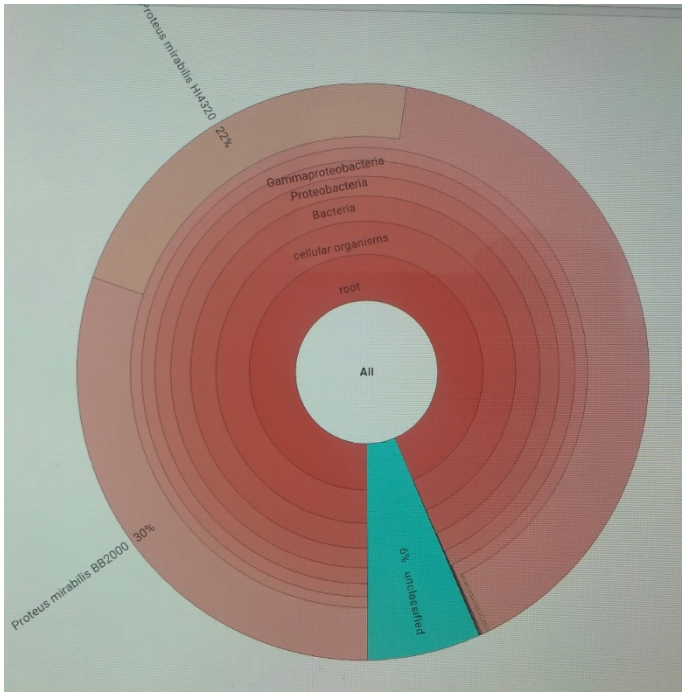

Figure 4: Krona Classification Chart indicating the relevant genomic sequencing data results for Proteus Mirabilis.

Antibiotic Susceptibility:

Antibiotic tablets selected for antibiotic susceptibility for *P. mirabilis*, Figure 4, were

Amikacin (AN-30), Cefazolin (CZ-30), Cefoperazone (CFP75), Gentamicin (GM-10), Oxacillin (OX-1), Tobramycin (NN-10), Trimethoprim (TMP-5), and Vancomycin (VA-30).

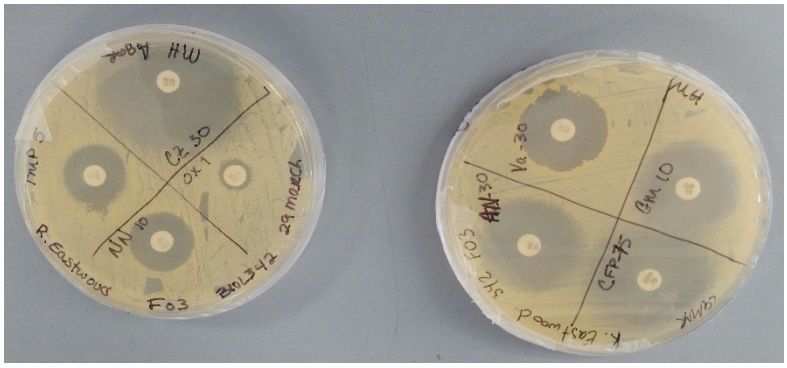

Figure 4: Antibiotic susceptibility diameters for AN-30, CZ-30, CFP-75, GM-10, OX-1, NN-10, TMP-5, and VA-30.

P. mirabilis was susceptible to every antibiotic, Table 1, except for Oxacillin in which it shared a value of 10mm with the zone diameter interpretive standards for resistance. The antibiotic that showed the highest susceptibility among the others was Cefazolin, with a diameter value of 40mm, indicating its potency and potential to eliminate a large amount of *Proteus mirabilis*. Cefoperazone showed the second highest susceptibility with a diameter value of 34mm.

Table 1: Antibiotic Susceptibility Results for *Proteus Mirabilis*.

Antibiotic	**Resistant (mm)**	**Intermediate (mm)**	**Susceptible (mm)**	***P. mirabilis* measurement (mm)**	**Results R/I/S**
Amikacin AN-30	<14	15-16	>17	27	S
Cefazolin CZ-30	<14	15-17	>18	40	S
Cefoperazone CFP-75	<15	16-20	>21	34	S
Gentamicin GM-10	<12	13-14	>15	27	S
Oxacillin OX-1	<10	11-12	>13	10	R
Tobramycin NN-10	<12	13-14	>15	19	S
Trimethoprim TMP-5	<10	11-15	>16	21	S
Vancomycin VA-30	<14	15-16	>17	24	S

Cefazolin would be the most efficient antibiotic to use on my bacterial isolate because it has a susceptibility diameter that is 22mm larger than the susceptibility minimum of 18mm. Cefazolin is a cephalosporin used to treat bacterial infections and is used most commonly for

urinary tract infections and staphylococcus infections. The antibiotic works by preventing cross-linking in peptidoglycan in Gram-positive and Gram-negative bacterial species and by inactivating penicillin binding proteins (Antimicrobe.org).

Discussion:

While using the Gram-staining technique, I was able to identify my bacteria as a Gram-negative species. Physiological testing of my isolate was very inadequate. Even after the duplication of API20E test strip, my results were still inconclusive. In my initial interpretation of my first failed test, I had concluded that I may not have introduced a large enough bacterial sample to the required medium. Therefore, I added extra bacteria to the medium for the repeat test. My results had changed slightly, but not enough to give me any conclusive results for positive identification for any type of bacterial species. Genome sequencing indicated my bacterial strain was *Proteus mirabilis*, in which the API20E test should have been the best option to use. The fluid thioglycollate test concluded my species was a facultative anaerobe which was further supported by the genome sequencing and bioinformatics testing. My data failed to support my original hypothesis. I hypothesized I would isolate a Gram-positive, anaerobic bacterium common to the microbial community found on dog's footpads and in between their toes. I originally did not have any idea what bacterial species I would find and I wasn't aware of different physiological characterizations until after we conducted the lab experiments. However, *Proteus mirabili*s is a very intriguing species that has been researched extensively.

Proteus mirabilis is a Gram-negative, facultative anaerobic species of bacteria that are commonly found in soil, water, intestinal tracts of mammals, and the human microbiome. They have the tendency of becoming pathogenic if they are introduced into the urinary tract, lungs, or open wounds. When they are introduced into the urinary tract, the bacteria may induce an infection, especially in hospital patients that are undergoing long-term catheter usage. Flagella provide *P. mirabilis* with efficient motility and swarming capability. In order for these bacteria to enter the urinary tract, they may form stones, protease, iron and zinc acquisition, urease, or even other toxins. Hydrogen sulfide is a bi-product commonly produced in these motile, Gram-negative, rod-shaped bacteria that are most closely related to *E. coli.* Other diseases associated with *P. mirabilis* include, urosepsis, urinary stones, asymptomatic bacteriuria, cystitis, pyelonephritis, empyema, osteomyelitis, and neonatal meningoencephalitis (Schaffer, J, et al. 2016).

Biofilm formation is important for the virulence of *P. mirabilis*, which have been known to form crystalline biofilms on surfaces of abiotic urinary catheters. Catheter associated urinary tract infections have an increased rate of mortality and morbidity in patients affected. The process of inserting a urinary catheter into a bladder facilitates entry of bacteria not commonly found in the urinary tract. Once these uropathogenic bacteria have entered the urinary tract, their adhesion mechanisms recognize uroepithelial cells as binding sites to which they can produce a protective biofilm made of exopolysaccharides that prevents damage from host cells and antibiotics. Crystalline biofilms are the product of urease production in which urease catalyzes urea and forms carbon dioxide and ammonia. As urease catalyzes urea, urine pH is increased and struvite and apatite crystals are formed. The formation of ammonia is toxic for uro-epithelial cells and can damage them (Fusco, A. et al. 2016).

BB2000 and HI4320 are two strains of *P. mirabilis* associated with urinary tract infections that can induce full body symptoms when a patient is infected. Overall, there are several strains of this bacterium. Some of the strains affect the digestive tract; the two mentioned

affect the urinary tract; yet, others are even non-virulent (Xiaolu Shi, et al. 2016). These two strains accounted for 52% of the sequenced bacterial isolate genome obtained from my tri-colored border collie's right, front, foot pad.

In general, *P. mirabilis* has been known to be susceptible to Beta-lactamase/Beta-lactam inhibitors and cephalosporins. However, by the 1900's, a few strains of this bacterium were noted to have developed resistance to Beta-lactam by acquiring Beta-lactamase with the acquisition of Beta-lactamase (Jann-Tay, W., et al. 2014).

P. mirabilis is found in a variety of locations in abundance and therefore, exposure to this species tends to be widespread. It can become pathogenic if introduced into the urinary tract, which can have long term negative effects on those who require a catheter. The biofilms that are formed, allow the bacteria to thrive, and are difficult to remove once situated. Therefore, eradicating *P. mirabilis* pathogenic strains entirely from urinary tracts can be quite costly, and not to mention, hard or even impossible to do. The bacteria can be found in a non-pathogenic state within the human microbiome, so interaction with your pup is not very likely to increase rate of urinary tract infections unless your urinary tract is damaged, allowing bacteria to enter.

The bacterial isolate from my dog's foot contained 52% of two pathogenic strains of *Proteus mirabilis*. Further research on whether transmission of these microbes would be likely or detrimental to susceptible parties, such as the elderly or the immunocompromised that may require long term use of urinary catheters, would be recommended. Many elderly people have dogs, and those dogs, just as people, are exposed to environmental soils and water that contain *P. mirabilis*.

References

1. Coskun, Yesim, et al. "A Newborn with Hemorrhagic Meningoencephalitis Due to Proteus Mirabilis." Drug Discoveries & Therapeutics, vol. 10, no. 6, Dec. 2016, pp. 334-337. EBSCOhost, doi:10.5582/ddt.2016.01068

2. Fusco, Alessandra, et al. “Biofilm Formation and Immunomodulatory Activity of Proteus Mirabilis Clinically Isolated Strains.” *International Journal of Molecular Sciences*, vol. 18, no. 2, Feb 2017, pp. 1-11. EBSCO*host* doi: 10.3390/ijms18020414

3. Gimmler, Jacqueline R., et al. "Determining Canine Skin Concentrations of Terbinafine to Guide the Treatment of Malassezia Dermatitis." Veterinary Dermatology, vol. 26, no. 6, Dec. 2015, pp. 411-e96. EBSCOhost, doi:10.1111/vde.12245

4. Khan, M. Azizur R., et al. "Artificial Receptors for the Electrochemical Detection of Bacterial Flagellar Filaments from Proteus Mirabilis." Sensors & Actuators B: Chemical, vol. 244, June 2017, pp. 732-741. EBSCOhost, doi:10.1016/j.snb.2017.01.018

5. Jann-Tay, Wang, et al. "Antimicrobial Susceptibilities of Proteus Mirabilis: A Longitudinal Nationwide Study from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) Program." BMC Infectious Diseases, vol. 14, no. 1, Jan. 2014, pp. 486-10. EBSCOhost, doi:10.1186/1471-2334-14-486

6. Morozova V, Kozlova Y, Tikunova N, et al. “Lytic bacteriophage PM16 specific for Proteus mirabilis: a novel member of the genus Phikmvvirus.” Archives of Virology [serial online]. September 2016;161(9):2457-2472. Available from: Academic Search Premier, Ipswich, MA. Accessed March 22, 2017

7. Mueller, Ralf S., et al. "A Review of Topical Therapy for Skin Infections with Bacteria and Yeast." Veterinary Dermatology, vol. 23, no. 4, Aug. 2012, pp. 330-e62. EBSCOhost, doi:10.1111/j.1365-3164.2012. 01057.x

8. Pierezan, Felipe, et al. "The Skin Microbiome in Allergen-Induced Canine Atopic Dermatitis." Veterinary Dermatology, vol. 27, no. 5, Oct. 2016, pp. 332-e82. EBSCOhost, doi:10.1111/vde.12366

9. Schaffer, J.N., Pearson, M.M. “*Proteus mirabilis* and Urinary Tract Infections.” Microbiol Spectr. October 2015; 3(5). Available from: Department of Microbiology, New York University School of Medicine, New York, NY. doi:10.1128/microbiolspec.UTI-0027-2013.

10. Shi, Xiaolu, et al. "Comparative Screening of Digestion Tract Toxic Genes in Proteus Mirabilis." Plos ONE, vol. 11, no. 3, 24 Mar. 2016, pp. 1-12. EBSCOhost, doi:10.1371/journal.pone.0151873.

11. Stich, Ashley N., et al. "Clinical Efficacy of Low-Level Laser Therapy on Localized Canine Atopic Dermatitis Severity Score and Localized Pruritic Visual Analog Score in Pedal Pruritus Due to Canine Atopic Dermatitis." Veterinary Dermatology, vol. 25, no. 5, Oct. 2014, pp. 464-e74. EBSCOhost, doi:10.1111/vde.12144

12. Wei, Ting, et al. "Persistence of Antibiotic-Resistant and -Sensitive Proteus Mirabilis Strains in the Digestive Tract of the Housefly (Musca Domestica) and Green Bottle Flies (Calliphoridae)." Applied Microbiology & Biotechnology, vol. 98, no. 19, Oct. 2014, pp. 8357-8366. EBSCOhost, doi:10.1007/s00253-014-5846-9

13. Weese, J. Scott. "The Canine and Feline Skin Microbiome in Health and Disease." Veterinary Dermatology, vol. 24, no. 1, Feb. 2013, pp. 137-e31. EBSCOhost, doi:10.1111/j.1365-3164.2012.01076.x

14. Wilks, Sandra A., et al. "Novel Insights into the Proteus Mirabilis Crystalline Biofilm Using Real-Time Imaging." Plos ONE, vol. 10, no. 10, 30 Oct. 2015, pp. 1-13. EBSCOhost, doi:10.1371/journal.pone.014171