Porphyromonas endodontalis and Alzheimer's Disease: Characteristics and Genomics

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The human mouth is a diverse and systematic area of the body. It can be the first line of defense in preventing certain things from entering the body, it begins the process of digesting food, and is responsible for one of the 5 major senses. It's microbiome contains nearly 6 million bacteria, that are made up of 700 different species, The flora of the mouth can also contain other microbes including fungus, protozoa, mycoplasma, and viruses.
Maintaining a healthy environment in the mouth is important in maintaining good hygiene and can possible help prevent disease and maintain health in other parts of the body [1]. The *Porphyromonas* genus of bacteria is a microbe that can be a very present microbe in the mouth. There are different species in the *Porphyromonas* genus, for example *P. gingivalis* or *P. endodontalis* [2]. *P. endodontalis* is a microbe that is commonly found in infected endodontic areas, or what are commonly known as root canals. *P. gingivalis* is a microbe that is commonly present in individuals the suffer from periodontitis which is commonly known as gum disease [3]. More and more studies of microbes of the mouth have been completed because of the data being found, *P. gingivalis* is one of these microbes.

It is being studied heavily because of a protein that the microbe produces called gingipains. These gingipains have been found in the brains of individuals that suffer from Alzheimer's disease. These gingipains are coded by genes RgpA, RgpB, and Kgp that exist in the genome of *P. gingivalis and P. endodontalis* [4]. These gingipains are then transported with the assistance of a pore secretion system protein coded by the gene porU. Studies haven't been able to pinpoint the direct effect of these gingipains to Alzheimer's but their presence alone has been surprising [5]. It could possibly just be that since Alzheimer patients become much less active and are unable to take care of themselves that there dental hygiene begins to decline, and since it is not as much of an importance periodontitis may be more likely and that is why the gingipains are seen in their brains. Alzheimer's disease affects around 5.8 million Americans, and the effects of the disease not only affect sufferers but significantly affects the loved ones also. Researchers are intrigued and want to try to discover the effect of these gingipains, and if there are possible ways to both combat *P. gingivalis* or the gingipains specifically. Since *P. endodontalis* contains these same genes and is from the same genus, but is lower in bio safely level, which makes it a more ideal microbe to work with.

MATERIALS AND METHODS

General Lab:

Gloves were used to prevent contamination of samples and provide protection from the tested microbe. When microbes were cultured in either broth or on plates a Bunsen burner, sterile plate spreaders, or inoculating loops were used to transfer microbes to the new form of media. All mediums of culturing were either sterilized, disposed of in hazardous waste, or stored in walk-in freezers in the biology department.

Experimentation:

To begin, research was completed in order to determine how to anaerobically culture *P. endodontalis* both in a broth and eventually on a plate. Then a sample was ordered from ATCC and was received in the form of a freeze-dried pellet. The pellet was then removed from a glass conical and placed into a vial of mixed meat media using tweezers to transfer the pellet.Once intermixed with the media, it was incubated in an agitating incubator at 37º C.

Figure 1: *Mixed Meat Media tubes used to incubate samples throughout summer.*

Once cultures were cloudy enough in broth cultures, blood agar plates was cultured using a sample from the broth cultures. Once the broth was spread on the blood agar plates they were incubated

in the GasPakEZ container system with two anaerobic sachets again at 37º C.

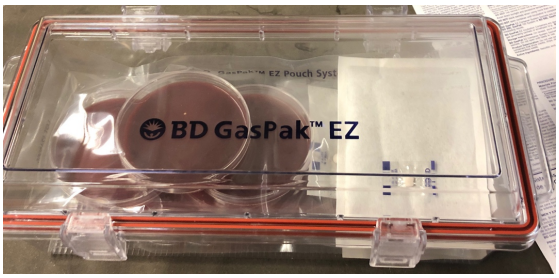


Figure 2: *GasPak EZ container with satchets and plates ready for incubation*

Since there was a difficulty with culturing *P. endodontalis*, a method was used to help increase concentrations of the cultures. This method began with the transfer of the colony from the blood agar plate to a new mixed meat media using a pipette tip which was left in the tube with the microbe on it. Then once the broth appeared cloudy enough new blood agar plates had broth spread on them and placed in the GasPak EZ container and incubated at 37º C until growth was observed.

Finally, when enough cultures were grown antibiotic testing began. Seven different antibiotic discs were tested, they were Ampicillin, Amoxicillin, Chloramphenicol, Erythromycin, Penicillin, Tetracycline and Vancomycin. The plates were created by first using an equal volume of mixed meat media broth on each plate, the broth was spread out as evenly as possible the one plate had four of the seven antibiotic discs placed on it and the other had the remaining three discs placed on it. The plates were then incubated in the GasPak EZ container at 37º C until growth was observed.

To move forward, dental plaque was extracted from the mouth using dental floss. The dental plaque was then spread on both a blood agar plate and a LB plate so it could be grown be aerobically and anaerobically. Once they plaque was spread both plates were incubated at 37º C and the blood agar plate was incubated in the GasPak EZ container. When growth was observed the culture from the LB plate was then transferred to a tube with LB broth using a pipette tip and the culture from the blood agar plate was transferred to a tube of mixed meat media. Once both broths looked cloudy antibiotic resistance was tested using the same 7 antibiotics used on P. endodontalis. The aerobic culture was incubated at 37º C and the anaerobic culture was placed in the GasPak EZ container at the same temperature.

After Kirby-bauer test to determine antibiotic resistance for P. endodontalis and both cultures of dental plaque was complete genomic DNA isolation was completed. To begin this process, a volume of each of the cultures broth was used in a DNeasy ultraclean microbial kit from Qiagen. Once the DNA was isolated, PCR was used to amplify a specific region of DNA in each of the samples. These regions were specified by using forward and reverse 16s rRNA primers. After the PCR reaction, the samples were placed on a gel to determine if the samples contained the fragment of interest. After the fragments were confirmed by examining the gels, the fragments of interest were isolated from the gel and other PCR samples using a gel/PCR DNA fragment extraction kit. Once these fragments were isolated, they were then prepared to be sent to Eurofins for nucleotide sequencing. Once the sequencing was returned, the data was examined to determine what microbes were grown from the *P.endodontalis* culture and both the aerobic and anaerobic dental plaque cultures.

RESULTS

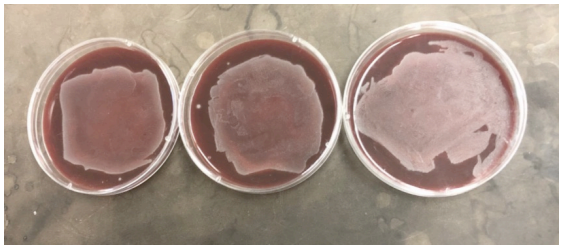


Figure 3: *First set of blood agar plates, cultured from original mixed meat media containing freeze dried pellet.*

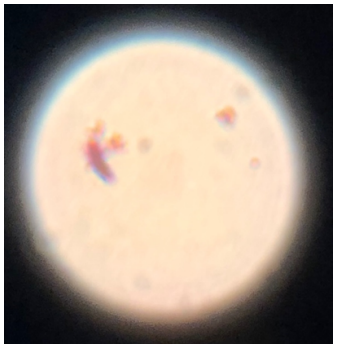
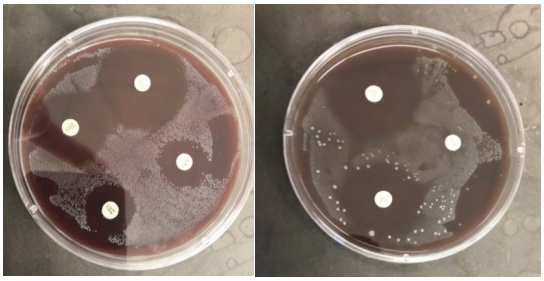


Figure 4: *Microscopic evidence of a gram stained microbe from the culture on the plates in Figure 3.*

Cultures observed in Figure 3 confirmed that a microbe was cultured from the freeze-dried pellet placed in the mixed-meat media tube seen in Figure 1. Literature examined said that *P. endodontalis* would be a gram-negative rod-shaped microbe, so the image seen in figure 4 insinuated that the correct microbe was being cultured because the microbe observed was pink, gram negative, and rod shaped.



Figures 5 and 6: *Kirby-Bauer plates completed on P. endodontalis.*

KIRBY BAUER TEST RESULTS	
AMPICILLIN	Susceptible
AMOXICILLIN	Susceptible
CHLORAMPHENICOL	Susceptible
ERYTHROMYCIN	Resistant
PENICILLIN	Susceptible
TETRACYCLINE	Susceptible
VANCOMYCIN	Susceptible

Table 1: *Antibiotics and their status of resistance to P. endodontalis.*

Zones of inhibition were observed for all the antibiotics tested with *P. endodontalis* except for erythromycin. Tetracycline and ampicillin had the largest observed zones of inhibition.

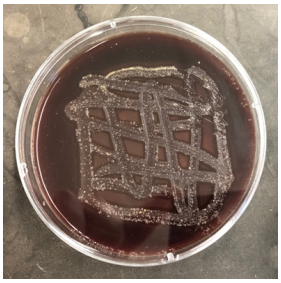
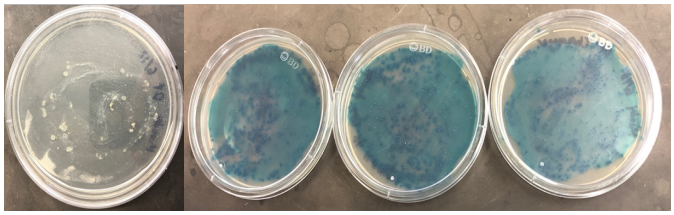
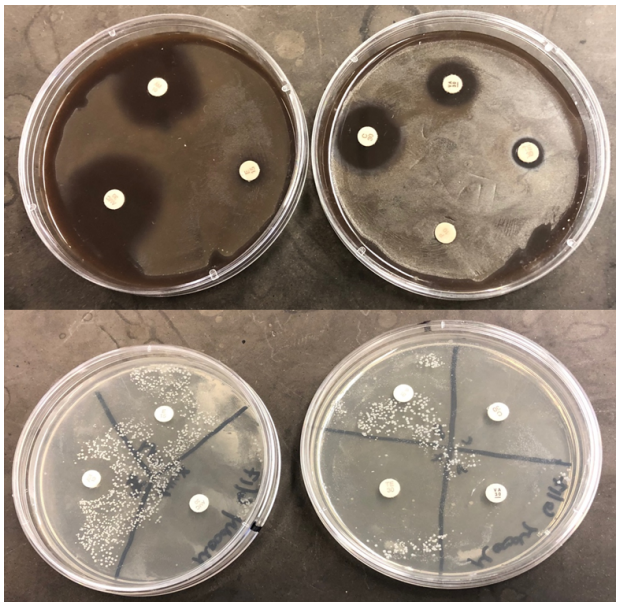


Figure 7: *Anaerobic Dental Plaque Culture*



Figures 8 and 9: *LB plate of aerobic dental plaque; CHROMagar plates of aerobic dental plaque*

Figures 7 and 8 show that the samples taken from the dental floss did culture into a microbe after being incubated. Figure 9 helped ensure that there was only one microbe cultured on the LB plate used for the aerobic dental plaque culture.



Figures 10 & 11: *Kirby-Bauer test plates of anaerobic & aerobic dental plaque.*

ANAEROBIC KIRBY BAUER TEST RESULTS	
AMPICILLIN	Susceptible
AMOXICILLIN	Susceptible
CHLORAMPHENICOL	Susceptible
ERYTHROMYCIN	Resistant
PENICILLIN	Resistant
TETRACYCLINE	Susceptible
VANCOMYCIN	Susceptible

Table 2: *Antibiotics and their status of resistance to anaerobic dental plaque culture.*

AEROBIC KIRBY BAUER TEST RESULTS	
AMPICILLIN	Susceptible
AMOXICILLIN	Susceptible
CHLORAMPHENICOL	Susceptible
ERYTHROMYCIN	Susceptible
PENICILLIN	Resistant
TETRACYCLINE	Susceptible
VANCOMYCIN	Susceptible

Table 3: *Antibiotics and their status of resistance to aerobic dental plaque culture.*

Like *P. endodontalis*, most of the antibiotics tested for both the anaerobic and aerobic cultures showed zones of inhibition. The anaerobic culture didn't have any zone of inhibition for erythromycin and penicillin. The biggest zones were ampicillin and amoxicillin. The aerobic culture didn't have a zone of inhibition for penicillin only.

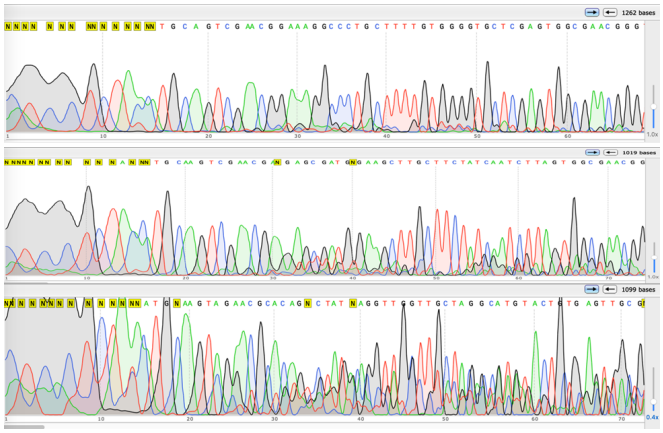


Figure 12: *Chromatogram data of DNA sequence of P. endodontalis, anaerobic dental plaque, and aerobic dental plaque*

Chromatograms of each of the three samples prove that each sample from the 3 different cultures were a microbe and that their DNA was successfully isolated.

DISCUSSION

Throughout the summer there was many results that were very positive at the time and created the idea that there were successful products of our work. For example, when gram staining of the *P. endodontalis* culture was seen as a gram negative microbe. Research and literature had shown that *P. endodontalis* was a rod shaped gram negative microbe, so at that point it was assumed that the correct microbe was growing. Also the positive results of all the Kirby-Bauer test, of different antibiotics, was very reassuring because of the positive data received from each of the tests. However, once genomic DNA was isolated from the cultures, amplified, and purified then sent for sequencing, the data showed that it was not actually *P. endodontalis* that was actually being cultured. A different anaerobic microbe of the mouth flora called *Veillonela parvula*. Which is both a gram negative and rod shaped microbe that is a very prominent microbe of most humans mouth flora. After receiving these results from the sequencing previous data from the summer was linked to these results, for example the stained microbe from figure 4, which likely reiterates the idea that the culture was more likely *V. parvula,* which has similar characteristics to *P. endodontalis*.

There are a couple different reasons and instances during the summer that could have affected growths of certain cultures or affected the results of some of the tests. One of the first things that comes to mind of an effect on test results was the creation of samples for sequencing. The results of sequencing were interesting, the sample for *P. endodontalis* returned as *V. parvula* and the anaerobic sample returned as an unidentifiable bacteria clone. There is a suspicion that the sample tubes could have possibly been mixed up. This is not a definite answer but a possibility. Another reason that the samples might not have been the best to having sequencing work done is that when the sample for each of the cultures was being purified, the first purification was completed by using the buffer provided by the purification kit, however the samples needed to be washed with distilled water instead so the purification process had to be repeated to create the right samples, and since they were purified twice the concentration of DNA was likely decreased pretty significantly.

Even with the struggles and differing results of data the summer was still extremely useful. The effectiveness of anaerobic culturing that was discovered and will be used by other students in the biology department for experiments to come. This summer was also very useful in understanding experiment design and timelines. This research could be expanded in the future by testing resistance of many other antibiotics. It could also be expanded in the future by completing different genomic work on both the porU gene or the different RgpA, RgpB, and Kgp. This different research would focus more on the specific proteins coded by these genes rather than the whole microbe itself, but is still an extremely viable area of research that this project could evolve to.

REFERENCES

Floy E. Dewhirst. The Oral Microbiome: Critical for Understanding Oral Health and Disease. California Dental Association. 2016. (44): 409-447

AJ van Winkelhoff, TJ van Steenbergen, J. de Graaff. Department of Oral Microbiology, Academic Center for Dentistry Amsterdam, The Netherlands. 1992. (9):431-4.

Colden, I. (n.d.). Porphyromonas endodontalis. Retrieved from https://microbewiki.kenyon.edu/index.php/Porphyromonas_endodontalis

Imamura T. Division of Molecular Pathology, Department of Neuroscience and Immunology,

Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan. 2003. 74(1):111-8.

Dominy, S. S., Lynch, C., Ermini, F., Benedyk, M., Marczyk, A., Konradi, A., . . . Potempa, J. Cortexyme, Inc., 269 East Grand Ave., South San Francisco, CA, USA. Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland….Department of Pharmacology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand. 2019. 5(1).

Oliviera de Machado, JF Siqueira Jr., GB Alves, R Hirata Jr., AF Andrade, Department of Dentistry, Estácio de Sá University, Rio de Janeiro, RJ, Brazil. 2000. (12):729-32

Cao H, Qi Z, Jiang H, Zhao J, Liu Z, Tang Z. Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Sciences, State Key Lab of Transducer Technology, Shanghai, People's Republic China. 2012. (8):773-81.

Bedran, T. B., Marcantonio, R. A., Neto, R. S., Mayer, M. P., Grenier, D., Spolidorio, L. C., & Spolidorio, D. P. (2012). Porphyromonas endodontalisin chronic periodontitis: A clinical and microbiological cross-sectional study. Journal of Oral Microbiology, 4(1), 10123. doi:10.3402/jom.v4i0.10123