Investigating the Antibacterial Properties of *Micropterus salmoides* Mucus and Growing an *Escherichia coli* Biofilm in the Hogan-Rehak Apparatus

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Urinary catheters are commonly placed in patients to remove urine from the bladder. While this is a very important function, urinary catheters also cause infections of the urinary system. In fact, catheter associated urinary tract infections (CAUTI) are the most common hospital acquired infection (1). The overall health care costs caused by these infections are sizable given how often urinary catheters are used in acute care settings, extended care facilities, and in persons with injured spinal cords (2).

The primary reason that these catheters cause an infection in the urinary tract is something known as a biofilm that forms on the catheter. A biofilm is a slender, slimy film of bacteria that sticks to a surface. Most biofilms will form through a series of steps. The first step is the exposure of the catheter to urine. When this occurs a host of nutrients that the biofilm will need is deposited such as proteins, electrolytes, and other organic molecules (3). This is commonly referred to as the conditioning film. These nutrients adhere to the catheter via van der waals interactions and hydrogen bonds (4). With this structure in place, the necessary nutrients needed for bacterial growth are present and the antiadhesive properties of the catheter have been compromised (3). Next, the free swimming bacteria present in the urine can begin to colonize on the catheter through electrostatic and hydrophobic interactions as well as through the use of flagella (3). Once the bacteria has attached, it begins to divide, secrete an extracellular matrix, and recruit additional planktonic bacteria (3). The cycle is complete when individual organisms move off of the biofilm and contaminate the urine with pathogens (3). Once this has occurred, the patient containing the urinary catheter is at risk for developing a CAUTI.

There have been many attempts at preventing this biofilm formation using various antimicrobial agents. This project would attempt to use fish mucus examples extracted from *Micropterus salmoides*. Fish have developed advanced immune systems allowing them to not be taken over by the bacteria in their aquatic environments. One of the most important components of their innate immune system is the mucus they secrete. This mucus system has shown promise to resist bacteria formation in aquatic environments (5). Therefore, the goal of this experiment is to examine the viability of using *Micropterus salmoides* as a biofilm preventative for urinary catheters.

Multiple factors were considered when selecting *Micropterus salmoides* as the biofilm preventive agent. One of the main factors that was considered was the evidence showing fish mucus as an effective antibacterial agent. Research was conducted showing the presence of histones in the mucus (5). These histones have shown to contain significant bactericidal activity which could potentially deter the formation of biofilm (5). Additionally, ubiquitin has been identified in the surface mucus of many fish (5). Extracellular ubiquitin has been shown to play a significant role in preventing microbial invasion in systemic circulation on biological surfaces (5). This could potentially work in preventing the initial colonization of bacteria on a catheter surface. Other antimicrobial agents such as hemoglobin subunits, nucleoside diphosphate kinase and cofilin have also been identified in fish mucus samples (5). These additional agents have also been shown to possess significant antibacterial activity which further adds validity to the idea that *Micropterus salmoides* mucus possesses viability as a biofilm preventive agent (5). This mucus also resists biofilm formation on fish in an aquatic environment with a varying flow rate due to the fish movement. These conditions are somewhat similar to those found in a urinary catheter with the flow rate and aquatic environment being the overlap. Therefore, *Micropterus salmoides* mucus potentially possess multiple proteins that could make it a very effective biofilm preventative.

Materials and Methods For Antibacterial Screening of Mucus

Fish were obtained using a traditional line and hook method. The fish were handled using nitrile gloves, and a sterilized microspatula was used to scrape a small sample of the mucus from the dorsal side of the fish. The mucus samples were placed in a connocle tube and stored at 4ºC (6).

Three different methods were used to prepare the mucus into various extracts (8). One method was preparing a PBS extract, and the other methods involved preparing an acetic acid extract. For the PBS extract, the mucus was centrifuged at 10,000 rpm for 10 minutes. Following this, the supernatant was mixed in a 1:1 ratio with phosphate-buffered saline and tested against using a Kirby-Bauer test against seven different common bacteria and fungi commonly found in a CAUTI: *Pseudomonas, S. aureus, B. thuringiensis, E. coli, C. albicans, A. flavus, S. cerevisiae*.

The second extract synthesized was an acidic extract. The mucus was mixed with 3% acetic acid in a 1:1 ratio. Next, the mixture was homogenized through sonification. Following this, the mucus was put in a hot water bath and then placed in an ice bath for approximately 15 minutes. The resulting extract was then centrifuged at 18,000 rpm for 30 minutes. The supernatant was taken and filtered through a syringe. The resulting acetic acid extract was tested against the same seven bacteria and fungi through the use of a Kirby-Bauer test.

The final method extract used the method as the acetic acid extract explained previously, but an additional step was added. After filtering the supernatant through the syringe, the resulting supernatant was vacuumed overnight to remove any additional water in order to increase the concentration of the desired protein content in the sample. The content was then resuspended in 10 µl of phosphate-buffered saline. The resulting extract was tested against a single bacterium, *B. thuringiensis,* using a Kirby-Bauer test. More samples of this extract were desired but limited availability of mucus prevented them from being obtained. A Bradford protein assay was used to determine the protein concentration of the two acetic acid samples. The results are summarized in the chart below.

Mucus Extract	Protein Concentration (mg/ml)
Unvacuumed Acetic Acid Extract	.076
Vacuumed Acetic Acid Extract	.219

Materials and Methods for Developing an *E. Coli* Biofilm

The Hogan Rehak biofilm formation apparatus, pictured below, was constructed using a balloon catheter and a separatory funnel. The balloon catheter was attached to the output of the separatory funnel. *E. coli* inoculated synthetic urine was cultured for seven days and then ran through the separatory funnel and into the catheter at a fixed rate of 42 ml/hr. Following this, the catheter was cut open and gently washed with water to remove any urine or *E. coli* cells that were not part of the biofilm matrix. The catheter was then stained with methylene blue and examined under a microscope. This image can be examined in Figure 2.

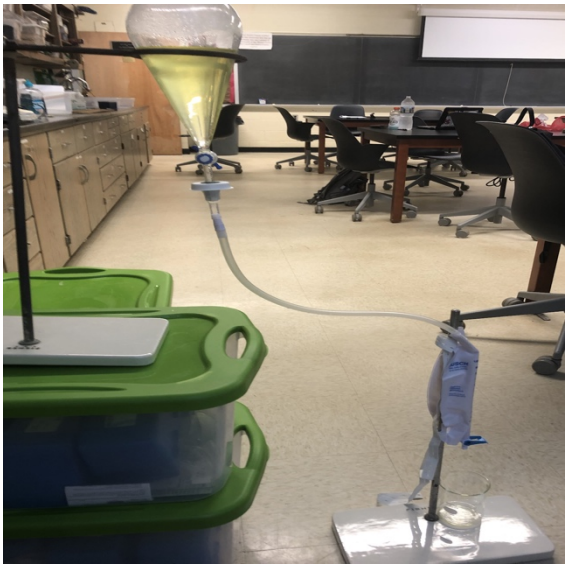

***Figure 1**: Hogan-Rehak Biofilm Formation Apparatus*

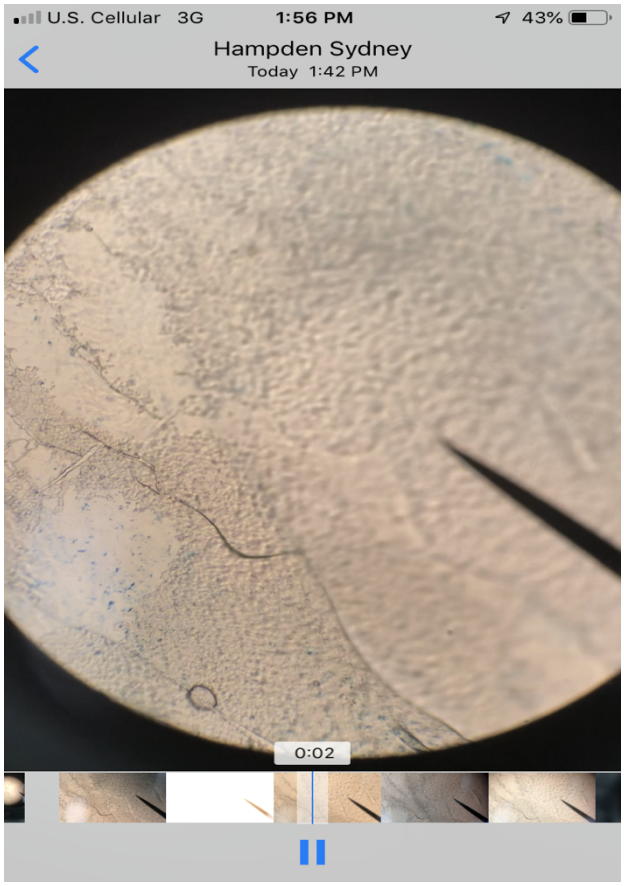

Figure 2: *Stained E. coli Biofilm From Urinary Catheter*

Results

The PBS mucus extract produced no zone of inhibition for any of the bacteria or fungi it was tested against on the Kirby-Bauer test. Similarly, the acetic acid extract produced no zone of inhibition for any of the bacteria or fungi it was tested against. After the vacuumed acetic acid extract was tested against *B. thuringiensis,* it produced a significant zone of inhibition. The image of this can be seen in figure 3. After the stained lumen of the catheter was examined under a microscope, it was evident that a very immature biofilm had formed. This can be examined in figure 2 where there are a series of stained *E. coli* cells located at the bottom left of the image.

Analysis

Based on the results, the PBS extract and the unvacuumed acetic acid extract lacked the protein concentration needed to produce an antibacterial agent. This resulted in consistently negative results for the Kirby-Bauer tests against various bacteria and

Figure 3: *Positive Kirby-Bauer Test from Vacuumed Acetic Acid Extract*

fungi. It is suspected that the vacuumed acetic acid extract contained enough of the isolated peptide product from the mucus that it was able to successfully resist *B. thuringiensis*. The proteins responsible for this antibacterial resistance are mostly likely due to the presence of the isolated glycoproteins (8). The biofilm pictured in figure 2 was determined to be a very immature biofilm. An important point in making this determination was the fact that even after that lumen of the catheter was rinsed with deionized water the *E. coli* cells were still adhering to the catheter. This adherence is the first step in the formation of a mature biofilm. This new biofilm formation apparatus can now be used in the undergraduate classroom as a simple way to construct a viable biofilm.

Conclusion

The overall goal of the project was to determine if *Micropterus salmoides* mucus had any antimicrobial properties, and if so, could it prevent biofilm formation in urinary catheters. Three mucus extracts were prepared and tested against seven different bacteria and fungi. The results were all negative except for the only test the vacuumed acetic acid extract was used for. The vacuumed acetic acid extract could not be tested against all forms of bacteria and fungi strains but did exhibit significant antibacterial properties when tested against *B. thuringiensis*. The Hogan-Rehak biofilm formation

apparatus did manage to successfully produce an immature *E. coli* biofilm in approximately ten days.

Future Work

The next phase of this specific research will be to apply the mucus to sections of the catheter and see if it deters the formation of biofilm. Other areas of interest will be to collect enough quantity of mucus to test the vacuumed acetic acid extract against the other six forms of bacteria and fungi. The science of using fish mucus for various applications is fairly new and is very exciting. There are several unique avenues future scientists could take the work we completed this summer.

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REFERENCES

Jordan, R. P., Waters, M. G., Stickler, D. J., & Williams, D. W. (2014, February 01). Biocide Activity against Urinary Catheter Pathogens. Retrieved from https://aac.asm.org/content/58/2/1192

Kumar, N. P., Marimuthu, K., Rao, R. V., Xavier, R., Kathiresan, S., Suresh, C., & Sreeramanan, S. (2012). Antimicrobial activity of different tissues of snakehead fish Channa striatus (Bloch). *Asian Pacific Journal of Tropical Disease, 2*. doi:10.1016/s2222-1808(12)60170-4.

Nigam, A. K., Kumari, U., Mittal, S., & Mittal, A. K. (2015). Evaluation of antibacterial activity and innate immune components in skin mucus of Indian major carp, Cirrhinus mrigala. *Aquaculture Research, 48*(2), 407-418. doi:10.1111/are.12889.

Saint, S., & Chenoweth, C. E. (2003). Biofilms and catheter-associated urinary tract infections. *Infect Clin Dis North Am* 2003 Jun;17(2):411-32.

Singha, P., Locklin, J., & Handa, H. (2017). A review of the recent advances in antimicrobial coatings for urinary catheters. *Acta Biomaterialia, 50*, 20-40. doi:10.1016/j.actbio.2016.11.070

Soto, SM. “Importance of Biofilms in Urinary Tract Infections: New Therapeutic Approaches,” Advances in Biology, vol. 2014, Article ID 543974, 13 pages, 2014. https://doi.org/10.1155/2014/543974.

Tran, K., Rivas, T., & Dowell, G. (2018). Proteomic Profiles of *Lepomis macrochirus, Micropterus salmoides* and *Pomoxis nigromaculatus.* Hampden-Sydney College Biology Department.

Trautner, B. W., & Darouiche, R. O. (2004). Role of biofilm in catheter-associated urinary tract infection. *American journal of infection control*, *32*(3),177-83.