Evaluating a Zebrafish Model to Determine The Etiology of Epitheliocystis

Mitchell Morin, RoseAnn Vik, Wilmore Webley, PhD

Department of Microbiology University of Massachusetts Amherst

Abstract

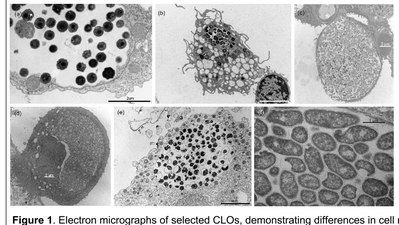
Epitheliocystis is a severe disease affecting the gills and skin of fish that can lead to high rates of mortality. Infection has been documented in over 90 species of both freshwater and marine fish. Epitheliocystis is characterized by development of cysts in the gill epithelia and may lead to the fusion of gill lamellae [1]. The etiology, transmission, and epidemiology of epitheliocystis remains largely unknown. It is believed that the causative agent of this disease comes from a family of pathogenic, intracellular bacteria, that form vacuoles that closely resemble those of the human pathogens from the Chlamydiaceae family. We hypothesize that *Chlamydia*-like organisms (CLO), or environmental chlamydiae, are an important etiologic agent of epitheliocystis.

We developed a zebrafish model to determine susceptibility to infection, mode of transmission and factors that influence the disease. Two *Chlamydia*-like organisms, *Waddlia chondrophila* and *Simkania negevensis,* were used to model the pathogenic intracellular bacteria, while zebrafish (*Danio rerio)*, exposed/infected using different models. We used polymerase chain reaction to determine the presence of microbe in the gills of infected fish.

Our data shows that zebrafish can become infected with W. chondrophila, however, the dose or inclusion forming units that was used did not lead to widespread mortality.

Background

Advances in molecular techniques have led to the discovery of new Chlamydiae, which belong to novel families in the Chlamydiae phylum. These families include: *Parachlamydiaceae, Simkaniaceae, Rhabdochlamydiaceae, Waddliaceae, Candidatus Piscichlamydiaceae*. In this study, we used *W. chondrophila* and *S. negevensis*. These organisms, along with other CLOs have a distinctive developmental cycle, alternating between two different physiological forms, the elementary bodies (EBs) and reticulate bodies (RBs) [2, 3]. EBs are the infectious form of Chlamydiae and other CLOs, which have reduced metabolic activity, while RBs are the metabolically active and are the replicative form of the organism [4]. CLOs are often pathogenic to humans and many animal species, including fish, birds and mammals. For the past 30 years, they have been identified in the aquatic environment as endosymbionts of amoeba and seaworms and are currently believed to cause respiratory diseases among fish, including epitheliocystis [5].



Candidatus Parilichlamydiaceae, *Candidatus Clavichlamydiaceae*,and *Candidatus Actinochlamydiaceae* have all been isolated from the gills and other tissues of diseased fish. However, there is no definitive proof that they are responsible for the disease pathology [6].

Figure 1. Electron micrographs of selected CLOs, demonstrating differences in cell morphology of EBs and RBs. *Parachlamydia acanthamoeba* (a) in *A. polyphaga* (15 000 × magnification), *W. chondrophila* (b) within a macrophage at 16 h post-infection (4500 × magnification), *Ca.* Clavichlamydia salmonicola (c) and *Ca.* Piscichlamydia salmonicola (d) in gill epithelial cells of Brown trout, *E. lausannensis* (e) within *Acanthamoeba commandonii* at 48 h post-infection (4500 × magnification) and *Ca.* Syngnamydia venezia (f) in gill epithelial cells of broad-nosed pipefish. Scale bars are shown in individual images. Note the diversity in EB and RB morphologies among different CLOs and the number of chlamydial cells in each inclusion [6].

2. Methods

Culture of *W. chondrophila:* *Acanthamoeba castellanii* , an environmental amoeba, is a potential reservoir for CLOs. We grew *A. castellanii* in culture at room temperature in conical flasks. *W. chondrophila* was cultured in *A. castellanii* for approximately 48-96 hours. This was then harvested after the desired time point. Before infecting the zebrafish, most of the stock of *W. chondrophila* was pooled and then aliquoted into 2 mL vials to ensure that all vials that were used would have the same or similar IFU (Inclusion Forming Units), which is a measurement of infectivity.

Infection of Zebrafish: Before any infections were started in our zebrafish model, the fish were allowed to acclimate to their new environment to limit as much stress to the fish as possible. The fish were separated into 4 different tanks, Tanks 1 and 2 being control tanks, while Tank 3 would be infected with 1 mL of the pooled *W. chondrophila* stock, and Tank 4 would be infected with 2 mL of the pooled *W. chondrophila* stock. Tanks 1 and 2 held 15 fish each, while Tanks 3 and 4 held 5 fish each. The fish were fed daily, besides the day before infection. The *W. chondrophila* was added straight to the water with the filter in the tanks turned off for approximately 30 minutes.

PCR & Gel Electrophoresis:

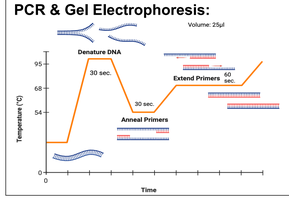


Figure 2 : Polymerase Chain Reaction (PCR) was used to amplify any *W. chondrophila* DNA found in the fish samples by using specific primers and temperatures. [7]

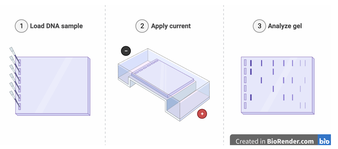


Figure 3: The process of Gel Electrophoresis was used to separate our DNA fragments that were amplified during PCR to show the presence of *W. chondrophila.* [7]

3. Results

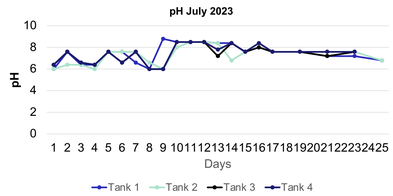


Figure 4: pH levels of water from July 1st- July 16th. The average pH of each tank varied over time as the fish adjusted and we tried to normalize it by adding specific agents. However, the fish did not show any observable changes in behavior.

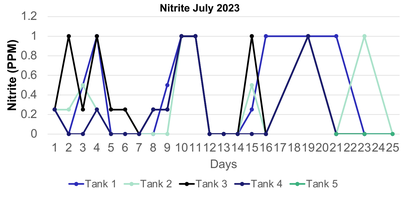


Figure 6: Nitrite levels of water from July 1st-July 16th. The average nitrite level of each tank varied. Each tank spiked at different timepoints. Tanks 1 and 2 were similar due to the having 10 more fish than Tanks 3 and 4. This could also be due to the variation in the amounts of nitrifying bacteria that are found in the tanks, which were being used for the first time.

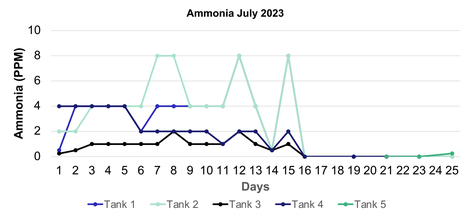


Figure 5: Ammonia levels of water from July 1st-July 16th. Tank 2 showed a significant spike between days 6-16. This was due to overfeeding of Tank 2 along with the number of fish that were present in the tank. To account for the rise in ammonia, we used a water conditioner along with lowering the amount of food that was put into the tank each day. The drop in ammonia level between days 16-25 is also due to a change in testing kits.

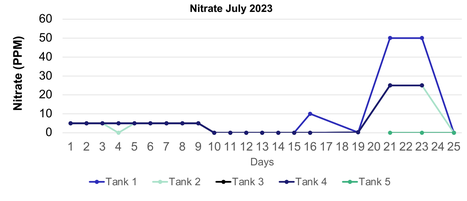


Figure 7: Nitrate levels of water from July 1st-July 16th. The average nitrate levels in the tanks remained constant for much of the testing period. However, we measured variations in tanks 1 and 3 between days 19-25.

4. Results

Figure 8: Gross examination of an infected fish. Example of fish showing potential signs of infection 10 days after *W. chondrophila* was added to the tank. Histological analyses will be used to confirm this.

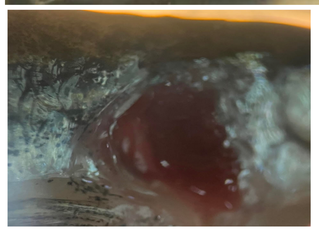


Figure 9: Dissection of a fish with signs of epitheliocystis. The operculum was removed, exposing the gills which displayed a darker color than expected and patches that could indicate significant disease, compared to other fish that showed no pathology.

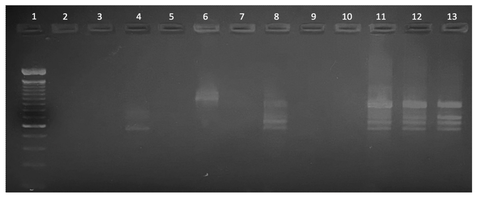


Figure 10: PCR from of zebrafish from infected tank. The fish was dissected, and we examined the different body parts for the presence of *W. chondrophila* using a 16S PCR primer pair. The agarose gel image shows the presence of chlamydial DNA in the gills, tail, abdomen and head, but not in the operculum or in the water sample.

Samples:
1-100 bp ladder
2-*S.negevensis*
3-Amoeba
4- Amoeba infected with *W. chondrophila*
5-Tank 3 water
6-Tank 4 water
7-F2T2 Operculum
8-F2T2 Gills
9-F1T2 Operculum
10-F1T2 Gills
11-F1T4 Tail
12-F1T4 Abdomen
13-F1T4 Head

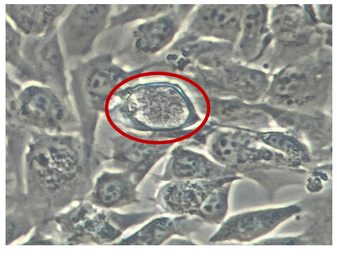


Figure 11: *W. chondrophila* cultured in Glial Cells. This figure shows a phase contrast image of a 36h inclusion (circle) inside an infected glial cell. This large vacuole contains multiple EBs/RBs, with the potential to infect hundreds of new cells upon release. Most of the surrounding cells in the field remain uninfected.

5. Future Work

- ❑ Determine the optimal inclusion forming units of *W. chondrophila* that will lead to overt pathology of epitheliocystis in this model
- ❑ Evaluate the factors that increase the risk/probability of infection and disease in this zebrafish model

6. References

[1] Blandford MI, *et al.*: Epitheliocystis in fish: An emerging aquaculture disease with a global impact. *Transbound Emerg Dis* 2018, 65(6):1436-1446.
[2] Stelzner, K, et al.: Intracellular lifestyle of Chlamydia trachomatis and host–pathogen interactions. *Nat Rev Microbiol* 21, 448–462 (2023)
[3] Di Pietro, et al.: Chlamydia trachomatis and Chlamydia pneumoniae Interaction with the Host: Latest Advances and Future Prospective. *Microorganisms* 2019, 7, 140.
[4] Clemente Tatiana M., et al.: Establishing the intracellular niche of obligate intracellular vacuolar pathogens. *Frontiers in Cellular and Infection Microbiology* 2023, 13: 2235-2988
[5] Blandford MI, *et al.*. Epitheliocystis in fish: An emerging aquaculture disease with a global impact. Transbound Emerg Dis. 2018 Dec;65(6):1436-1446. doi: 10.1111/tbed.12908. Epub 2018 May 23
[6] Pawlikowska-Warych M, Deptuła W. Characteristics of chlamydia-like organisms pathogenic to fish. J Appl Genet. 2016 Feb;57(1):135-41. doi: 10.1007/s13353-015-0303-8. Epub 2015 Jul 10.
[7] Biorender.com

We thank our sponsors and supporters

United States Department of Agriculture
National Institute of Food and Agriculture

UMassAmherst
College of Natural Sciences
Center for Agriculture, Food, and the Environment