STUDIES TO IMPROVE THE CONTROL OF VIRULENT *Aeromonas hydrophila* AND EVALUATE THE IMPACT OF ENVIRONMENTAL FACTORS ON ITS ABUNDANCE IN CATFISH AQUACULTURE PONDS

Reporting Period
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Funding Level

Year 1	$212,848
Year 2	$161,634
Total	$374,482

Participants

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PROJECT OBJECTIVES

Objective 1. Determine the environmental factor(s) and animal vector(s) that are correlated with epidemic *A. hydrophila* abundance and dissemination.
- a. Determine VAh abundance in relation to in-pond environmental factors.
- b. Determine correlations between VAh abundance and biological, physical and chemical factors over time.
- c. Evaluate potential for animal dissemination of VAh.
- d. Develop VAh population dynamics models in naïve and endemic ponds.

Objective 2. Determine the disinfection method(s) that will allow removal of *A. hydrophila* from seines
- a. Establish quantification methods to detect bacteria in seines.
- b. Test disinfection protocols under laboratory conditions.
- c. Assess seine disinfection methods under pilot-field conditions.

Objective 3. Determine the efficacy of vaccine and/or probiotics delivered orally in preventing mortality due to *A. hydrophila* in farmed catfish.
- a. Evaluate vaccine delivery by feed.
- b. Evaluate vaccine and/or probiotic delivery by feed.
- c. Conduct a pond-scale trial of vaccine and probiotic prophylaxis.

ANTICIPATED BENEFITS

A highly virulent and clonal population of *Aeromonas hydrophila* is the causative agent of an ongoing epidemic of motile Aeromonas septicemia in farmed catfish. Originally with an epicenter in western Alabama, this disease epidemic has now spread to Mississippi and Arkansas. This research will help us understand the environmental and human factors that contribute to its spread, develop effective disinfection and management practices that can result in improved biosecurity, and develop control measures for farms afflicted with this epidemic.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS

Objective 1: *Determine the environmental factor(s) and animal vector(s) that are correlated with epidemic A. hydrophila abundance and dissemination.*

Subobjective 1a: *Determine VAh abundance in relation to in-pond environmental factors. –and-*
Subobjective 1b: *Determine correlations between VAh abundance and biological, physical and chemical factors over time.*

Mississippi State University

Year 1

We have recruited 4 catfish operations (1 in the Delta Region, 3 in East Mississippi) to participate in this project. In June, we sampled 4 ponds from Farm #1 in East Mississippi, 3 of which had a history of VAh. We collected pond water (for molecular, physical and chemical analysis), pond sediment, benthic oligochaetes, zooplankton, as well as kidney, gill and lower intestines from resident fish (n=12 fish/pond). The sampling effort was reasonable, but sample processing proved highly labor intensive and time consuming. Many samples required long-term storage (-80°C) until they could be evaluated.

In September, a more sweeping sampling endeavor took place to identify candidate ponds for future long-term sampling. A total of 28 ponds were sampled from 4 different operations, 1 in the Delta, and three in East Mississippi. As before, we collected pond water for molecular, physical and chemical analysis, pond sediment, benthic oligochaetes (D. digitata, chironomidae, and pools of mixed oligochaetes), and zooplankton. Based on earlier samplings, whole blood, gill and rectal swabs were collected in lieu of kidney biopsies, as they are non-lethal (sampled fish can be returned to the ponds), less time consuming, and comparably informative. On Farm #1, the same 4 ponds were sampled as in June. On Farm #2 (East Mississippi) we sampled 10 ponds, 6 of which had no history of VAh, and 4 of which had overcome VAh outbreaks earlier in the summer. Similarly, on Farm #3 (East Mississippi) we sampled 2 ponds, both of which had recently recovered from VAh outbreaks. On Farm #4 (Mississippi Delta), we sampled 6 ponds with active outbreaks of VAh and 6 ponds with no prior history of VAh. Again, the sampling effort was reasonable, but sample processing is highly labor intensive and time consuming. Many samples have been placed in long-term storage (-80°C) until they could be evaluated.

In addition, samples were collected from Farm #4 (Mississippi Delta) in October. Farm #4 was chosen as all samples had not been processed yet and Farm #4 had active outbreaks during the September sampling. It should be noted, all outbreaks on Farm #4 had resolved by the October sampling. Nearly 1,000 samples have been collected for biological, chemical, physical and molecular analysis from ponds from multiple VAh categories (Active VAh outbreak, recovered from VAh outbreak, History of VAh and No history of VAh). This sample processing and analysis is currently underway and will serve to identify a panel of 12 pond candidates (6 VAh ponds, 6 negative ponds) for continued long-term sampling. In addition, the results of this initial sampling will identify which variables, if any, are uninformative and can be excluded from future sampling to better streamline this process.

Preliminary analysis has detected VAh from pond water, pond sediments, Dero digitata, chironomids and resident fish collected from ponds with and without active VAh outbreaks. This suggests these sampling protocols are sufficient to detect VAh in the system. However, until all analysis is complete, any correlations between pond factors and the presence of VAh in the production system would be premature.

Subobjective 1c: *Evaluate potential for animal dissemination of VAh.*

Mississippi State University and USDA-NWRC

Year 1

In previous research we have shown that VAh survives the through the GI tract of fish eating birds and viable VAh can be shed at substantial levels for a limited period when birds consume infected fish. We have initiated evaluations of potential pathways for VAh shed from predatory birds to become established in ponds. These pathways include colonizing invertebrates and fish. We have immersion exposed dero worms (*Dero digitata*) and larval midges (chronomids-*Chironomus dilutes*) to VAh. Initial results indicate that the dero worms did not become colonized but the chironomids appear to have taken up the bacterium. We are currently evaluating the minimal dose needed for chironomid colonization.

Objective 2: *Determine the disinfection method(s) that will allow removal of A. hydrophila from seines.*

Subobjective 2a. *Establish quantification methods to detect bacteria in seines.*

Auburn University

Year 1

A qPCR based has been set up using specific primers against *A. hydrophila*. The qPCR protocol uses a commercial SYBR master mix and after optimization the efficiency of the reaction is 88%. This is still slightly below acceptable levels for diagnostic (between 90-110%). However, the sensitivity of the protocols is below 50 fg per reaction, which is an acceptable level for

detection. The specificity of the reaction was tested with 12 fish pathogen species and proved to be specific for *A. hydrophila*.

We have also tested the ability of VAh to grow and form biofilm on seines (Figure 1). Cells attached to inert surfaces, including nylon and cotton seines, in less than 8 h but they did not exhibited the typical extracellular polymeric substances (EPS) characteristic of mature bacterial biofilms even when biofilms were allowed to develop for 48 h. We are currently standardizing the biofilm formation conditions to apply the qPCR method for quantifying attached cells.

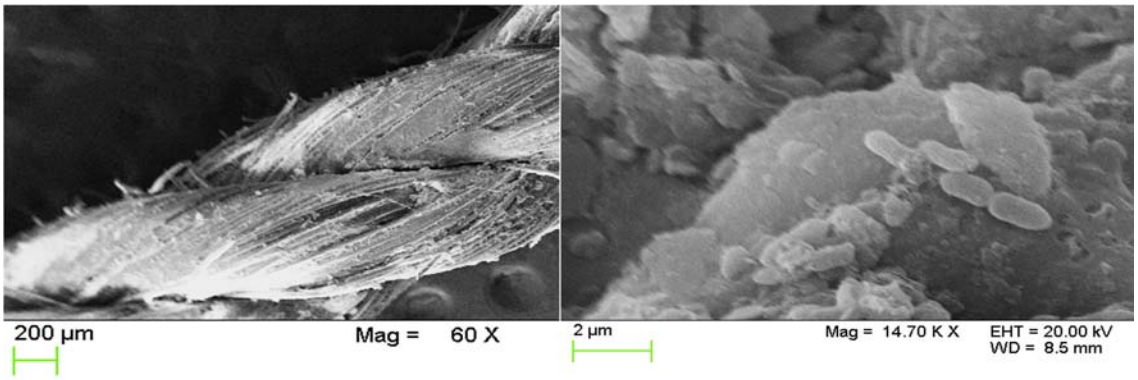

Figure 1. VAh cells attached on seine fibers examined under SEM.

Objective 3: *Determine the efficacy of vaccine and/or probiotics delivered orally in preventing mortality due to A. hydrophila in farmed catfish.*

Subobjective 3a. Evaluate vaccine delivery by feed.

Year 1

We have conducted studies to address oral delivery and to refine the vaccine candidate being used in these studies. First, to achieve biofilm-grown *Aeromonas hydrophila* for oral delivery to catfish, we used chitin flakes as a substrate for growth of VAh (in all studies the acronym VAh will refer to the use of the virulent *A. hydrophila* strain ML09-119) as a biofilm. While we were able to grow *A. hydrophila* chitin flake surface (data not shown), the size of the chitin flake was found to be too large to introduce into a catfish fingerling. For this reason we switched to using chitin powder as a substrate for VAh growth, since it is the same chemical substrate and the size was conducive to introduce by a gavage needle into a catfish orally. As with the chitin

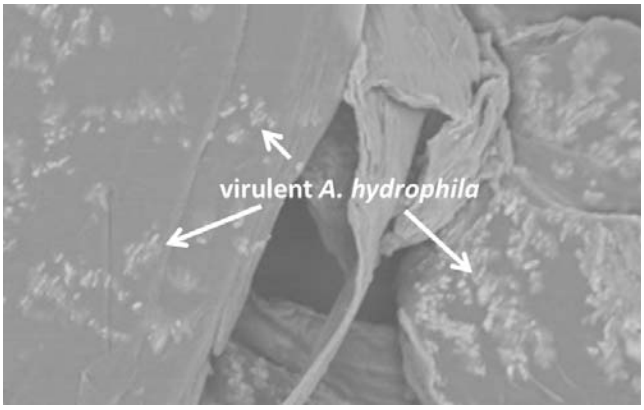

Figure 2. Scanning electron microscopy of virulent *A. hydrophila* ML09-119 grown as a biofilm on chitin

flakes, we grew VAh on chitin powder for biofilm formation, using static cultures at 30^{o}C for multiple days. We quantified VAh cells grown on the chitin powder by washing the chitin powder repeatedly with 1 x PBS, and then vortexed the VAh biofilm off of the chitin powder vigorously and then plated for CFU/ml. We could achieve approximately 10^5 CFU/mg of chitin powder after 3 days of incubation. In order to demonstrate that the VAh cells were associated with the chitin powder we visualized VAh biofilms using scanning electron microscopy (Fig. 1), which demonstrated the presence of VAh cells on the surface of the chitin powder. Because we could remove the vast majority of VAh cells from the chitin powder surface by vortexing as determined by culturing and SEM (data not shown), this likely indicates that the VAh cells will be removed from the chitin surface upon introduction into the catfish GI tract.

We evaluated the ability of VAh grown as a biofilm and introduced orally into a fingerling catfish to cause disease. In two different trials, using a range of VAh biofilm concentrations up to 10^7 CFU, we were not able to cause disease using the wild-type VAh. This is consistent with previous observations that oral gavage of VAh does not cause disease symptoms or mortality in fingerling catfish. It is unknown whether mature catfish, which seem more susceptible to disease in production ponds, would be susceptible to oral gavage of VAh grown in suspension or as a biofilm. Of course, the inability to cause disease by oral gavage is not critical to the success of Objective 3.1, since we plan to introduce the VAh vaccine orally and the ability to cause disease by wild-type is not essential. It is necessary for the VAh vaccine to be able to generate a significant immune response, and we are planning to introduce the VAh vaccine using multiple methods, including introducing bacterial cells in suspension, as a biofilm, and coated feed.

While the studies of biofilm-grown VAH were being conducted, we have also refined our selection of the VAh vaccine candidate. We have two attenuated VAh mutants, the *iolA* mutant first described in the SRAC project, and a *ymcA* mutant that is also completely attenuated in its virulence when introduced by IP injection into fingerling catfish. For both mutants we have been studying the mechanism(s) by which these mutations attenuate virulence, using USDA AFRI funding. These studies suggest that the *iolA* mutant is attenuated due to a polar effect on an upstream genetic locus *iolR*, which is known to encode a transcriptional regulator IolR that is involved in the regulation of virulence factors in *Salmonella* and other bacterial pathogens. The hypothesis is that the *iolA* mutation causes an increase in *iolR* transcription (from deletion of IolR-binding site(s) within the *iolA-iolR* promoter region) and that increased amounts of IolR repress the transcription of multiple virulence factors responsible for VAh pathogenesis. In contrast, the *ymcA* mutant is hypothesized to be attenuated due to a defect in O-antigen assembly. Since the *ymcA* mutant is a structural defect, and we have data that supports slightly better protection against wild-type VAh challenge by the *ymcA* mutant compared to the *iolA* mutant, we have decided to move forward with the *ymcA* mutant as the best vaccine candidate for the subsequent experiments in Objectives 3.1, 3.2, and 3.3. Furthermore, we have recently completed construction of a new *ymcA* mutant that is markerless, in other words the chloramphenicol resistance cassette that was in the mutant has been removed, allowing us to introduce an attenuated vaccine strain that does not have the presence of recombinant DNA within its genome. This may be helpful for regulatory approval and application of this vaccine. The trials of this vaccine by oral gavage are being conducted fall of 2014.

Subobjective 3b. *Evaluate vaccine and/or probiotic delivery by feed.*

Auburn University

Year 1

We plan to begin the trials of the vaccine with and without the probiotic *Bacillus amyloliquefaciens* strain AP193 after the challenges are completed in Subobjective 3a.

Subobjective 3c. *Conduct a pond-scale trial of vaccine and probiotic prophylaxis.*

Auburn University

Year 1

The construction of the in-pond raceway systems is being conducted during the winter months, and discussions are ongoing with catfish producers on location of the in-pond raceway systems. In addition, we have leveraged funding from the Alabama Agricultural Experiment Station to procure a 10L microbial fermentor in order to produce sufficient bacterial spores to conduct these in-pond studies. This will allow much more efficient spore production and allow greater scale of application. In addition, a pond study is being conducted currently at the N. Fisheries ponds at Auburn University to evaluate the growth performance of catfish being fed with *Bacillus* strain AP193. This study is using 4 replicate ponds in the control (no probiotic) and treatment group (catfish fed with 10^7 CFU per g of feed) for 10 weeks, with multiple environmental factors (water quality indicators, temp) and microbial community studies being determined. While no SRAC funding is being used for this pond study, and no VAH would be expected to be present at the Auburn University ponds, this study will be very important for regulatory approval prior to the introduction of probiotic bacteria into catfish feed in the summer of 2015. We plan to harvest catfish filets in 2 weeks and analyze these for the presence of metabolites known to be produced by AP193. These data together with the safety and growth performance data we have for the use of these probiotic strains will be important for our ability to apply bacterial spores in feed for a production pond.

IMPACTS

Mississippi State University has recruited four catfish operations for participation in this study. Nearly 1,000 samples have been collected for biological, chemical, physical and molecular analysis from ponds from multiple VAh categories (Active VAh outbreak, recovered from VAh outbreak, History of VAh and No history of VAh). Preliminary analysis has detected VAh from multiple substrates, suggesting the proposed sampling protocols are sufficient to detect VAh in the system.

Auburn University has developed vaccine strains that ae being evaluated for their ability to protect catfish against virulent *Aeromonas hydrophila.* A probiotic bacteria is being evaluated for its ability to promote the growth of catfish and prevent disease such as Motile *Aeromonas* Septicemia (MAS) caused by virulent *Aeromonas hydrophila*

PUBLICATIONS, MANUSCRIPTS OR PAPERS PRESENTED

None to date.