FINAL PROGRESS REPORT

Project Title	**Development of a phage-based diagnostic test for the rapid detection of pathogenic Vibrio species in bivalves**
Reporting Period	03/01/2017 to 02/28/2020
Author (Project Coordinator)	Name of person submitting this report. Helene Marquis
Key Word	Vibrio parahaemolyticus, bacteriophages, bivalves, luciferase, diagnostic
Funding Level	Total funds allocated for this project to date. *Year One: FY 2017/18, $ amount* $ 90,506 *Year Two: FY 2018/19, $ amount* $100,918 *Year Three: FY 2019/20, $ amount No cost extension*
Participants	List participating personnel and respective institutions/agency/business; include outreach representative. Indicate funded participants with an asterisk. **Name(s)/Role(s):** Helene Marquis (Project Coordinator)* Institution/Agency/Business: Cornell University Address(s): Dept. Microbiol. Immunol., Ithaca, NY 14853 Phone(s): 607-253-3273 Email(s): hm72@cornell.edu Funded (Yes/No): Yes **Name(s)/Role(s):** Martin Wiedmann (PI) Institution/Agency/Business: Cornell University Address(s): Department of Food Science Phone(s): 607-253-3393 Email(s): martin.wiedmann@cornell.edu Funded (Yes/No): No **Name(s)/Role(s):** Gregg Rivara (PI)* Institution/Agency/Business: Cornell University Cooperative Ext. of Suffolk County Address(s): 3690 Cedar Beach Road, Southold, NY 11971 Phone(s): 631-852-8660 x 35 Email(s): gjr3@cornell.edu Funded (Yes/No): Yes **Name(s)/Role(s):** Cheryl A. Whistler (PI)* Institution/Agency/Business: University of New Hampshire Address(s): Rudman Hall, Room 210, Durham, NH 03824 Phone(s): 603-862-2359 Email(s): Cheryl.whistler@unh.edu Funded (Yes/No): Yes **Name(s)/Role(s):** Stephen H. Jones (Collaborator) Institution/Agency/Business: University of New Hampshire Address(s): 114 James Hall, Durham, NH 03824 Phone(s): 607-862-5124 Email(s): shj@unh.edu Funded (Yes/No): No

	Name(s)/Role(s): Rod G. Getchell (Collaborator) Institution/Agency/Business: Cornell University Address(s): Dept. Microbiol. Immunol., Ithaca, NY 14853 Phone(s): 607-253-3393 Email(s): rgg4@cornell.edu Funded (Yes/No): No **Name(s)/Role(s):** Kari Brossard Stoos (Research Associate)* Institution/Agency/Business: Cornell University Address(s): Dept. Microbiol. Immunol., Ithaca, NY 14853 Phone(s): 607-253-4224 Email(s): kstoos@ithaca.edu Funded (Yes/No): Yes **Name(s)/Role(s):** Jennifer Ren (Technician) * Institution/Agency/Business: Cornell University Address(s): Dept. Microbiol. Immunol., Ithaca, NY 14853 Phone(s): 607-253-4224 Email(s): jr632@cornell.edu Funded (Yes/No): Yes **Name(s)/Role(s):** Marvin Ho (Research Assistant) * Institution/Agency/Business: Cornell University Address(s): Dept. Microbiol. Immunol., Ithaca, NY 14853 Phone(s): 607-253-4224 Email(s): mbh99@cornell.edu Funded (Yes/No): Yes **Name(s)/Role(s):** Kelly Sams (Technician)* Institution/Agency/Business: Cornell University Address(s): Dept. Microbiol. Immunol., Ithaca, NY 14853 Phone(s): 607-253-4224 Email(s): kle39@cornell.edu Funded (Yes/No): Yes

Project Objectives	*Objective 1. Isolate V. parahaemolyticus and V. vulnificus specific phages.* We hypothesize that bacteriophages that infect specifically *V. parahaemolyticus* or *V. vulnificus* and that are universal for all clinical isolates of these two bacterial species can be isolated. For this purpose, water samples collected from the northeast coast will be used to amplify phages specific to *V. parahaemolyticus* and *V. vulnificus*. These phages will be analyzed to identify those that are specific to clinical isolates of these two bacterial species. *Objective 2. Develop a bacteriophage-based diagnostic test for the detection of* virulent *V. parahaemolyticus and V. vulnificus*. We hypothesize that recombinant phages expressing GFP of *luxAB* will enable the rapid detection of virulent *V. parahaemolyticus and V. vulnificus* in bivalves. To reach this goal, the bacteriophages isolated in the first part of the project will be further characterized and the most infectious ones will be used for the development of a diagnostic test that will be specific, sensitive, cost-effective, and rapid.
Anticipated Benefits	State briefly how the project will benefit the aquaculture industry – directly or indirectly. Improving shellfish and finfish health maintenance, disease control, and biosecurity Development of a cost-effective assay for detection of aquatic animal pathogens in shellfish tissues and environmental samples
Project Progress	Summarize concisely for each objective the progress toward accomplishment to date. This has an 8,000 character limit. **New information since last report (2019) is typed in bold.** *Objective 1. Isolate V. parahaemolyticus and V. vulnificus specific phages.* We hypothesize that bacteriophages that infect specifically *V. parahaemolyticus* or *V. vulnificus* and that are universal for all clinical isolates of these two bacterial species can be isolated. For this purpose, water samples collected from the northeast coast will be used to amplify phages specific to *V. parahaemolyticus* and *V. vulnificus*. These phages will be analyzed to identify those that are specific to clinical isolates of these two bacterial species. Phage isolation. As reported last year, samples of seawater were collected at various locations along the coast in Rhode Island, Maryland and New York states

	during the summers of 2016, 2017, and 2018. These water samples were used to amplify and isolate phages that specifically infect *Vibrio parahaemolyticus (Vp).* Various clinical isolates of *Vp* were used to amplify and isolate phages. A collection of 18 phages capable of infecting *Vp* were isolated. All phages were isolated from New York State water samples. We have not been successful in isolating phages that can infect *Vv*. Phage purification. Each phage was purified by a series of single plaque isolation followed by amplification. This step was necessary to ensure the purity of each individual phage. Phage infectivity pattern. This step was performed to determine (i) whether these phages were different from each other (necessary to eliminate siblings) and (ii) whether these phages were selective for a subset of *Vp* isolates. The collection of 18 purified phages was tested against a collection of 43 *Vp* isolates and the pattern of infectivity (plaque formation) was recorded. Eleven distinct infectivity patterns were observed, suggesting that we have eleven distinct phage isolates. Each phage infected between five and twenty-seven isolates of *Vp*, none of them infected all isolates indicating a level of selectivity. Phage specificity. One of the major goal of this project is to identify phages that specifically distinguish virulent from avirulent isolates of *Vp*. Clinical isolates are presumed virulent, whereas environmental isolates can be virulent or avirulent. In the past, serotyping was used as a method to categorize isolates of *Vp*: antibodies specific to variable O (LPS) and K (Capsule) antigens. Although, this method has value, it is now recognized as a relatively imprecise approach to distinguish virulent and avirulent isolates of *Vp*. In more recent years, Multi Locus Sequence Typing (MLST) has been the method of choice to characterize isolates of Vp, although there is a growing interest in whole genome sequencing. Results from MLST indicates that three sequence types have been primarily associated with outbreaks of *Vp* infection in humans in the US: those are ST 3, ST 36, and ST 631. Of the 43 *Vp* isolates we are working with (17 clinical isolates, 10 environmental isolates, 16 unknown origin), ST information is available for only eight isolates (2 human and 6 environmental isolates). Considering the importance of determining which phages are specific to the most prevalent ST causing human infections, we need to know precisely the ST of every isolate we are working with. Therefore, we decided to determine the ST for all the isolates we are working with to better characterize the specificity of the phages we have isolated. **We have finalized the sequence typing and have identified the ST for the majority of the *Vp* strains we were working with.** *Objective 2. Develop a bacteriophage-based diagnostic test for the detection of* virulent *V. parahaemolyticus and V. vulnificus*. We hypothesize that recombinant phages expressing GFP of *luxAB* will enable the rapid detection of virulent *V. parahaemolyticus and V. vulnificus* in bivalves. To reach this goal, the bacteriophages isolated in the first part of the project will be further characterized and the most infectious ones will be used for the development of a diagnostic test that will be specific, sensitive, cost-effective, and rapid. Phage purification and characterization. We purified five phages by cesium chloride gradient. The selected phages have unique infectivity patterns and are more restricted to clinical isolates in their infection pattern. The purified phages were examined by electron microscopy. Their morphology suggest that they are all members of the Caudovirales order and Siphoviridae family. This family of phages is characterized by a non-enveloped icosahedral head, a noncontractile tail, and a linear double-stranded DNA genome. The genomes of these five purified phages were sequenced. Three of them are highly related despite having slightly different infection patterns. A fourth one

	is distinct, and the fifth phage needs to be re-sequenced due to low sequence coverage. The phages have genomes of 76-79 kb. Analysis of phage genomes using The RAST Server revealed that each phage has 105-108 coding sequences, most of them labeled as hypothetical proteins. Translated sequences were further searched individually using NCBI BLAST for proteins, resulting in the identification of a few enzymes and structural proteins. **Construction of recombinant phage: This part of the project has been the most challenging and we have not yet been successful. However, we have made progress and we are still working at it even though the funding has elapsed. Here is a summary of what we have done. We initially aimed at engineering recombinant phages with the selected reporter genes using a method called "rebooting synthetic genomes". However, after consultation with a molecular biologist and phage expert at Cornell, Prof. Joe Peters, we decided to follow a different approach. The next approach we considered consisted in introducing the reporter genes by allelic exchange into lysogens, which consist of phages whose genomes had integrated into the chromosome of a *V. parahaemolyticus* isolate. This approach was discarded after multiple unsuccessful efforts to isolate lysogens. The approach we are presently working at relies on introducing the reporter genes by allelic exchange in bacterial cells infected with the phage of interest. For this purpose, we obtained a plasmid (pCM18) that contains the entire lux operon, coding for the luciferase enzyme and its substrate. This plasmid had been engineered by Prof. James Caper from the University of Maryland, who provided us with the entire plasmid sequence. We cloned in this plasmid - upstream of the lux operon - the capsid-coding gene from one of the phages. We also cloned – downstream of the lux operon - the hypothetical gene that is downstream of the capsid-coding gene in the phage genome. This recombinant plasmid was electroporated into an isolate of *V. parahaemolyticus*. Vp transformants carrying this plasmid were isolated and infected with the phage. The expected outcome is recombination by allelic gene exchange between the plasmid and the phage, to generate recombinant phages that carry the lux operon whose expression will be under the control of the capsid promoter. We were in the process of screening for recombinant phages when we had to close the lab because of the Covid-19 pandemic. We will continue this project at a later time and published the results of this project within the next year.**
Accomplishments:	
Outreach Overview	Describe in general how your results have been extended to the intended users. OR, if they haven't yet, explain when & how this will occur. The project coordinator has been holding regular lab meetings to discuss results and troubleshoot experimental problems. Gregg Rivara (PI) has facilitated obtaining water samples in areas occupied by oyster farms along Long Island. Martin Wiedmann, and Cheryl Whistler have provided strains of Vp. Martin Wiedmann has also facilitated the acquisition of additional clinical strains from a collaborator. The PI, Helene Marquis, attended the Northeast Aquaculture Conference and Exposition in January of 2019, presenting a poster of this project. It was the intention of the PI to present this project to the American Fisheries Society Fish Health Section 2020 meeting this coming summer, but the meeting has been cancelled. This project will be presented at a relevant scientific meeting, hopefully within the next year.

Targeted Audiences	Provide information on the **target audience** for efforts designed to **cause a change in knowledge, actions, or conditions**. The target audience includes aquaculturists and food safety agencies.
Outputs:	Outputs are tangible, measurable products (website, events, workshops, products [AV, curricula, models, software, technology, methods, websites, patents, etc.], trainees, etc.). Do NOT include publications as they're listed separately. There are no outcomes/impacts yet for this project.
Outcomes/Impacts:	Describe how findings, results, techniques, or other products that were developed or extended from the project generated or contributed to an outcome/impact. **Outcomes/impacts are defined as changes in Knowledge, Action, or Condition.** 1. **Relevance:** Issue – what was the problem? Lack of an easy diagnostic test to assess contamination of oysters with virulent strains of V. parahaemolyticus 2. **Response:** What was done? Isolated and identified phages that selectively infect sequence types of Vp that cause foodborne infections in the Northeast USA 3. **Results:** How did your work make a difference **(change in knowledge, actions, or conditions)** to the target audiences? No outcome yet, as the test remains to be developed 4. **Recap:** One- sentence summary Phages that selectively infect sequence types of Vp that cause foodborne infections in the Northeast USA have been isolated and can be used to develop a diagnostic test that would be amenable to all entities from farm to table to improve food safety and prevent loss of revenues due to recalls.
Impacts Summary	Provide short statements (2-3 sentences) about each of the following: (pre-established fields for Researchers to complete short statement answers) 5. **Relevance:** Issue – what was the problem? Lack of a easy diagnostic test to assess contamination of oysters with virulent strains of V. parahaemolyticus 6. **Response:** What was done? Isolated and identified phages that selectively infect sequence types of Vp that cause foodborne infections in the Northeast USA 7. **Results:** How did your work make a difference **(change in knowledge, actions, or conditions)** to the target audiences? No outcome yet, as the test remains to be developed 8. **Recap:** One- sentence summary Phages that selectively infect sequence types of Vp that cause foodborne infections in the Northeast USA have been isolated and can be used to develop a diagnostic test that would be amenable to all entities from farm to table to improve food safety and prevent loss of revenues due to recalls.
Publications	Follow the format to list publications in the following categories:

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Presentations:

Oral: Infection and Immunity Seminars, Cornell University, December 2019
Posters: NACE, January 2019

Peer-reviewed:

Print (journal, etc.)
Digital (websites, videos, etc.)

Non-Peer-reviewed:

Extension factsheets
Popular articles

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<tr><td>Students/Participants:</td><td>
Provide the following information for every student that worked with you during the reporting period:

Name:
Whether Degree was completed during the reporting period (name, yes/no):
New or Continuing Student:
Capstone/Thesis Title (actual or anticipated):
Date of Graduation:
Provide link to thesis/dissertation document:
Name: Kari Brossard, PhD
Whether Degree was completed during the reporting period (name, yes/no): NA
New or Continuing Student: NA
Capstone/Thesis Title (actual or anticipated): NA
Date of Graduation: NA
Provide link to thesis/dissertation document: NA

Name: Jennifer Ren, undergraduate student
Whether Degree was completed during the reporting period (name, yes/no): Bachelor Degree in Animal Science, yes
New or Continuing Student: Hired after graduation as a technician to work on this project
Capstone/Thesis Title (actual or anticipated): NA
Date of Graduation: May 2019
Provide link to thesis/dissertation document: NA

Name: Marvin Ho, undergraduate student
Whether Degree was completed during the reporting period (name, yes/no): Bachelor Degree in Life Sciences, yes
New or Continuing Student: NA
Capstone/Thesis Title (actual or anticipated): NA
Date of Graduation: May 2019

Provide link to thesis/dissertation document: NA
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<tr><td>Partnerships</td><td>
List any partners that you worked with on your project. Provide the following information for each Partner:
<table>
<tr><th>Partner</th><th>Specific Type
Type</th><th>Level
Level</th><th>Nature of Partnership</th></tr>
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