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Development of monoclonal antibody-based assays for the detection of *Vibrio tubiashii* zinc-metalloprotease (VtpA)

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Running title –VtpA detection assays

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ABSTRACT

Vibrio tubiashii has been linked to disease outbreaks in molluscan species, including oysters, geoducks, and clams. In particular, oyster hatcheries in the Pacific Northwest have been plagued by intermittent vibriosis since 2006. Accurate detection of vibrios, including *V. tubiashii*, is critical to the hatcheries in order to allow for rapid remediation efforts. The current methods for detection of *Vibrio* spp. are not ideal for use at the hatchery. Plating samples requires time and is not sensitive to extracelluar pathogenic products, such as the secreted zinc-metalloprotease, VtpA. Other sensitive methods to detect bacteria, such as qPCR, require a high level of laboratory skills and expensive supplies that are prohibitive for use at hatchery sites. Thus, hatcheries would benefit from a sensitive, simple method to detect *V. tubiashii* and its secreted toxin. Here, we describe the development of two inexpensive and highly specific tests for the shellfish-toxic zinc-metalloprotease secreted by *V. tubiashii:* enzyme-linked immunoassays (ELISA) and a lateral flow immunoassay (dipstick assay). Both technologies rely on a set of monoclonal antibodies used in a sandwich format, with the capture antibody recognizing a different epitope than the detection antibody on the mature VtpA protein. Both assays are quantitative and give colorimetric readouts. The sandwich ELISA was sensitive when VtpA was diluted into PBS, but was markedly less sensitive in conditions that correlate with the environment of hatchery-derived samples, such as in the presence of seawater, algae, or oyster larvae. In contrast, the dipstick assay remained very sensitive in the presence of these contaminants, is less work-intensive, and much more rapid, making this format the preferred assay method for detecting VtpA on site in a hatchery or environmental setting.

Keywords

Vibriosis; ELISA; Dipstick Immunoassay; Hatchery; Detection

1.1 INTRODUCTION

World marine food farming production (aquaculture) is dependent on hatchery and nursery production of large quantities of high quality, disease-free larvae and juvenile animals. Bacterial diseases, particularly vibriosis, have been and continue to be a major cause of mortality in such hatcheries and nurseries, resulting in major losses and great expense for producers. This loss due to vibriosis has affected shellfish culture on all coasts of the U.S. and extends to other species groups including fish and shrimp. Vibriosis has been documented as a key impediment to hatchery and nursery culture of bivalve mollusks since the late 1970s (Brown, 1973, Brown, 1981, Brown and Losee, 1978, Elston et al., 1981), but outbreaks tend to be sporadic and severe. Although first identified as a cause of such mortalities in 1965 at U.S. East Coast locations, *Vibrio tubiashii* re-emerged in 2006 and since then is considered responsible for mass larval mortalities of Pacific oysters, Kumamoto oysters and geoduck clams causing production failures in West Coast states and resulting in a crisis in seed production in the region (Elston et al., 2008). A study of the 2006/2007 outbreak indicates that the impact of vibriosis is more severe than previously understood because low concentrations of pathogenic vibrio toxins---present more or less continuously--appear to be linked to early development defects in shellfish larvae (Elston, Hasegawa, Humphrey, Polyak and Häse, 2008). The reason for the re-emergence of *V. tubiashii* is not known but could be related to alterations in ocean currents and temperature regimes (Elston, Hasegawa, Humphrey, Polyak and Häse, 2008). Unfortunately, the intermittent nature of these outbreaks has failed to result in a substantial applied research focus on the disease for over three decades.

Our group has made substantial progress in identifying the basics of pathogenicity and toxicity of *V. tubiashii* over the last few years. We identified the structural genes of *V. tubiashii* that encode a metalloprotease (designated *vtpA*) and a hemolysin (designated *vthA*) (Hasegawa et al., 2008). Amino acid sequence analyses revealed that VtpA shares high homology with metalloproteases from a variety of *Vibrio* species, while VthA shows such homology only to the cytolysin/hemolysin of *V. vulnificus*. We then examined in detail the roles of the *V. tubiashii* extracellular hemolysin and protease in toxicity of culture supernatants to Pacific oyster larvae. We have concluded that the metalloprotease VtpA, but not the hemolysin VthA, is the critical factor for the toxicity of *V. tubiashii* supernatants on Pacific oyster larvae based on the following evidence: (i) treatment of *V. tubiashii* culture supernatants with metalloprotease inhibitors severely diminished the toxicity to Pacific oyster larvae, whereas other classes of protease inhibitors or a specific inhibitor of hemolysin did not affect the lethality; (ii) strains of *V. cholerae* expressing the *vtpA* gene, but not *vthA* or the vector plasmid, caused high larval mortality;

(iii) a VtpA-, but not VthA-, mutant strain of *V. tubiashii* showed a significant loss of toxicity to the oyster larvae; (iv) purified His-tagged protease, but not His-tagged hemolysin, caused larval toxicity (Hasegawa and Häse, 2009, Hasegawa, Lind, Boin and Häse, 2008). In addition, we found that closely related metalloproteases from several other *Vibrio* species are toxic to larval shellfish (Hasegawa et al., 2009) and have investigated the regulatory mechanisms underlying metalloprotease gene expression (Hasegawa and Häse, 2009). In summary, our work clearly showed the importance of the *V. tubiashii* secreted metalloprotease in shellfish disease. Thus, a rapid and sensitive detection method for this important and widely distributed toxic enzyme would greatly facilitate the development of effective intervention strategies to prevent shellfish larval mortality due to the metalloprotease commonly produced by *V. tubiashii* and other *Vibrio* spp. in hatcheries.

Once the presence of pathogenic *Vibrio* bacteria or their toxin can be confirmed in hatchery waters, such as incoming seawater, algae cultures, or larvae or juvenile shellfish tanks, treatments can be utilized to manage and reduce the impact of the disease. While additional refinement of water treatment methods will undoubtedly contribute to an overall solution, hatchery management experience and detailed research to date shows that the impact of marine hatchery vibriosis can be prevented in many cases, and greatly reduced in nearly all cases, if early detection is possible (Elston, Hasegawa, Humphrey, Polyak and Häse, 2008). This reduction has proven effective in commercial hatcheries by applying known effective management/sanitation methods and by providing knowledge for hatchery managers to enact production decisions based on the known risk of vibriosis exposure (Elston, Hasegawa, Humphrey, Polyak and Häse, 2008, Estes et al., 2004). Thus, the primary impediment today for marine hatcheries to make such management decisions is the lack of an on-site, real-time sensitive diagnostic method for the early detection of vibriosis. Diagnosis can be made with laboratory support, but involves a time lag of two to four days. The current method for detection of this pathogen relies on agglutination techniques using a polyclonal antiserum and does not allow for specific detection of the damaging toxins produced by the bacteria, nor is it amenable to the development of a fieldable method that could be widely used on-site. The development of a specific detection method usable in hatcheries, for the detection of *V. tubiashii* and its primary known toxigenic factors, would markedly advance the productivity and efficiency of marine hatcheries.

Here we report the development of antibody-based detection methods for the secreted toxin (a zinc-metalloprotease) produced by *V. tubiashii* that could facilitate the design of intervention strategies by making detection more widely available in a real time framework. As a result, such detection assays,

combined with effective management strategies, can be expected to effectively reduce or eliminate pathogen load from shellfish and marine hatcheries.

2.1 MATERIALS AND METHODS

2.1.1 Bacterial Strains and growth conditions.

Table 1.

All noted strains were streaked onto LB plates that contained 1% NaCl and incubated overnight at 30°C. 5 mL of LB broth supplemented with 1% NaCl was then inoculated with bacterial cells from the grown plate strains and incubated overnight at 30°C in a roller drum. Bacteria were then pelleted by centrifugation, the supernatant was filtered through a 0.22 µM filter, and was then frozen at -80° until use.

2.1.2 *V. tubiashii* zinc-metalloprotease production

Purified VtpA protein has already been used to demonstrate its toxicity towards oyster larvae (Hasegawa and Häse, 2009). Here, we produced larger amounts of purified His-tagged VtpA to generate monoclonal antibodies. Briefly, *E. coli* Top10 cells carrying the pBAD VtpA-His plasmid were grown in media containing arabinose (0.2% w/v) and ampicillin for several hours. Cells were pelleted by centrifugation upon reaching an OD600 of approximately 1.8, and frozen overnight. His-tagged proteins were harvested from the cell pellet using a Ni-NTA column (Qiagen) according to the manufacturer's instructions. Protein concentration was identified using the Bradford assay (Thermo Scientific), and proteolytic activity was determined using 1% azocasein as described previously (Hasegawa, Lind, Boin and Häse, 2008). His_6-VtpA was also expressed and purified by utilizing the commercially available services of GenScript Corp. (New Jersey, USA).

2.1.3 Development of monoclonal antibodies against VtpA

Monoclonal antibodies were produced and purified at the Monoclonal Core of the Vaccine and Gene Therapy Institute of Oregon Health and Sciences University (Beaverton, OR). BALB/c mice were immunized with His_6-VtpA, and antibodies were produced and purified using standard methods. Of 60 hybridoma lines produced, the monoclonal antibody 12F8 was determined to be optimal for the capture antibody, while 7C5, conjugated to biotin, was used for detection. The 7C5 antibody, unconjugated to biotin, was used in the dipstick detection assay.

2.1.4 Direct ELISA for VtpA

Serial dilutions of *V. tubiashii* His_6-VtpA (5 – 0.15625 µg/mL in PBS) were added to the wells of a polystyrene ELISA plate (Nunc MaxiSorp), and incubated at 37° for 1 hour. Plates were washed three

times with washing buffer (PBS, pH 7.4, 0.05% Tween-20), and unbound active sites were blocked with blocking buffer (1% BSA, PBS pH 7.4, 0.05% Tween-20) at 37° for 1 hr. The detection antibody 7C5-biotin was added to wells at a 1:200 dilution in blocking buffer, and the plates were incubated as before. The plates were washed, and 1 µg/mL NeutrAvidin in blocking buffer was added. After incubation (37°, 45 minutes) and washing, colorimetric detection was performed using ABTS (HRP Substrate Kit, Bio-Rad). The reaction was stopped with 2% (w/v) oxalic acid, and absorbance at 415 nm was read using a microplate reader (iMark, Bio-Rad) within 10 minutes.

2.1.5 Sandwich ELISA for VtpA

Polystyrene ELISA plates (Nunc MaxiSorp) were coated with the mouse anti-VtpA antibody 12F8 in PBS (pH 7.4) and were incubated for 1 hour at 37°. After incubation, plates were washed three times with washing buffer (PBS, pH 7.4, 0.05% Tween-20), and unbound active sites in the ELISA plate were blocked by blocking buffer (1% BSA, PBS pH 7.4, 0.05% Tween-20) at 37° for 1 hour. The plates were again washed, and antigen was added. Standard curves were prepared using serially diluted purified His_6-VtpA (5 – 0.15625 µg/mL). After incubation (37°, 1 hour), the plates were washed, and the detection antibody was added (1:200 dilution) in blocking buffer. The plates were again incubated and washed as before. 1 µg/mL of streptavidin-HRP (NeutrAvidin, Pierce) was added to wells and incubated at 37° for 45 minutes. After washing, colorimetric detection was then performed using ABTS (HRP Substrate Kit, Bio-Rad). The reaction was stopped with 2% (w/v) oxalic acid, and absorbance at 415 nm was read using a microplate reader (iMark, Bio-Rad) within 10 minutes.

2.1.6 Data Analysis

The lower limit of detection (LOD) of each assay was determined as the mean value of the blank (wells without protease) plus three times the standard deviation of the blank. The lower limit of quantification (LOQ) was determined as the lowest point on the relatively straight portion of the curve. Data was analyzed using Microsoft Excel, and the error bars shown represent the standard error of the mean.

2.1.7 Oyster Larvae and Hatchery Contaminants

Pacific oyster (*Crassostrea gigas*) larvae between 10 and 12 days old were provided by Coast Seafoods (Quilcene, WA). Seawater was also provided by Coast Seafoods from their Kona, Hawaii hatchery. Artificial seawater (ASW) was prepared from Instant Ocean (Aquarium Systems, Eastlake, OH), with the addition of Sea Buffer, Marine Stabilizer, and Marine Conditioner (Aquarium Systems) according to package directions. Final concentration of salt was between 28 and 30 ppm, and ASW was

filtered (0.2 µM pore size) before storage. Algae (PhytoPlex, Kent Marine) was used at a 1:10 dilution when noted.

2.1.8 Preparation of gold colloid/12F8 mAb Conjugation

Preparation of this reagent was performed as listed previously (Nadanaciva et al., 2009). Briefly, 40-nm gold colloid (Diagnostic Consulting Network, Carlsbad, CA) was brought from 4°C to room temperature and the pH was raised to 9 with 0.1 M K_2CO_3. 8 µg/mL of the antibody 12F8 was added to the gold colloid, and was immediately agitated on an orbital shaker at room temperature for 8 minutes. BSA was added (1% final concentration) and the tubes were again immediately agitated for 8 minutes, spun at 5,000 rcf at 4°C for 20 minutes, and supernatant was aspirated. The remaining gold/antibody conjugation was resuspended in 20 mM phosphate buffer (pH 7.4) + 10% BSA.The concentration of gold-conjugate was measured by optical density (SmartSpec Plus Spectrophotometer, Bio-Rad) at 530 nm, and was adjusted to an OD of 11. The conjugate was then stored at 4°C.

2.1.9 Creation of Antibody-spotted Dipsticks

Preparation of these dipsticks is as listed previously (Nadanaciva, Willis, Barker, Gharaibeh, Capaldi, Marusich and Will, 2009). Briefly, construction of dipstick cards required the following components: MIBA 010 Adhesive backing (30 cm length with 8 cm width cut to 6 cm); Millipore High Flow (HF) 120 nitrocellulose membrane (2.5 width and 100 m length cut in strips to ~32 cm), and Millipore cellulose fiber sample pads (CFSP, 20 cm x 30 cm, with 20 cm length cut to 3.7 cm). Adhesive backing was removed from the MIBA card and the HF 120 membrane was adhered along the bottom edge of the MIBA backing. The CFSP was adhered along the top edge of the MIBA backing, creating an overlap between the membrane and wick pad of 1 to 2 mm. A speedball roller was used to firmly adhere the CFSP to the adhesive backing. A paper cutter was used to cut ~4mm x 3.7 cm individual dipsticks, which were stored in a room temperature desiccator.

0.5 µg of monoclonal antibody 7C5 was spotted onto the lower 1/3 portion of the nitrocellulose membrane portion of each dipstick. For select dipsticks, 0.23 µg of a positive control spot (His_6-VtpA, GenScript Corp) was added in an equal volume in the upper third of the nitrocellulose membrane. Dipsticks were placed in a 37° C air incubator for 1 hour to dry the antibody and further adhere the wick pad and membrane to the adhesive backing. Dipsticks were then stored in a room-temperature desiccator for a minimum of 24 hours prior to use.

2.1.10 Dipstick Assays

Assay preparation is as listed previously (Nadanaciva, Willis, Barker, Gharaibeh, Capaldi, Marusich and Will, 2009). In a 96-well round-bottom microplate, 25 µL sample was combined with 25 µL

2X blocking buffer (MitoSciences, Eugene, OR). Extraction buffer (25mM HEPES, 100mM NaCl, pH 7.4) was used as a diluent as needed. 10 µl of gold-conjugated monoclonal antibody 12F8 was subsequently added. Dipsticks previously spotted with monoclonal antibody 7C5 were then added to each well, causing sample to wick up dipsticks. After 15 minutes, 40 µL Wash Buffer (150 mM NaCl, 50 mM Tris, pH 7.4) was added to each well and allowed to wick for 15 minutes. Dipsticks were then removed from wells, allowed to air-dry, and then results were examined visually.

3.1 RESULTS

3.1.1 Antibody production and screening

Monoclonal antibodies to His_6-VtpA were produced (60 in total) and preliminary examination of the specificity of each antibody by direct ELISA indicated that antibodies 7C5 and 12F8 would be ideal for use in ELISA. The data also indicated that the antibody 7C5 would be the best to use as a detection antibody (data not shown).

3.2.1 Direct ELISA

As rapid on-site detection is likely most useful for hatchery management, we determined the sensitivity of a simple direct ELISA for VtpA detection. We tested the ability of the 7C5 antibody to detect VtpA when the protease is in two different dilution agents – either PBS, or artificial seawater (ASW) in a direct ELISA format (Figure 1).

The sensitivity of the direct ELISA is increased at greater concentrations of VtpA, but detection in both PBS and ASW is limited at low concentrations of protease. In this format, the LOD is 0.05 µg/mL for both PBS and ASW, while the LOQ for PBS occurs at 0.625 µg/mL, and the LOQ for ASW as a diluent occurs at 1.25 µg/mL. This detection capability is not sufficiently sensitive in either diluent, so a sandwich ELISA was then developed.

3.3.1 Sandwich ELISA

To improve the detection capabilities of the ELISA format, a sandwich format was next tested. The antibody 12F8 was used to coat the surface of an ELISA plate before addition of antigen and detection using the 7C5 antibody as before. This format resulted in improved sensitivity of the assay to detect protease in PBS and ASW compared to the direct ELISA (Figure 2).

In the sandwich format, the LOD is 0.18 µg/mL, while the LOQ is 0.15 µg/mL for PBS. The LOQ falling below the LOD indicates that the LOD is the true limit of the assay. However, the sandwich ELISA shows increased sensitivity as compared with the direct ELISA, indicating that the initial step of antigen capture by a specific monoclonal antibody is advantageous.

Interestingly, a marked difference between VtpA diluted in PBS versu ASW is observed (Figure 2). The two diluents tested, PBS and ASW, differ significantly in their pH values. The pH of ASW is designed to be approximately 8, while the pH of PBS is 7.4. We hypothesized, therefore, that the pH change of the diluent affects the detection sensitivity of the sandwich ELISA. To test this, the pH of PBS was raised to that of ASW, and the pH of ASW was lowered to that of PBS. However, the pH change of each diluent to that of the other did not change the detection limit in each substrate (data not shown). Additionally, the use of a desalting column (Zeba Spin Desalting Column, 7K MWCO, Thermo Scientific) to perform a buffer exchange from protease diluted in ASW to PBS did not result in the same detection sensitivity as is seen in PBS (data not shown). Together, these results indicate that there are other differences that are the cause of the observed detection limit differences.

3.3.2 Effects of contaminants on sandwich ELISA

Samples from hatcheries will be present in an array of formats. It would be common to test for the zinc-metalloprotease in actual seawater, as well as algae growth tanks, and also tanks that are housing the growing oyster larvae and are being fed with algae. As such, these contaminants were tested in the direct ELISA format. Adding contaminants negatively affected the detection sensitivity of the assay (Figure 3). As before, detection was optimal with protease diluted in PBS, and was reduced when diluted in ASW. Adding contaminants to ASW, such as synthetic algae or oyster larvae, severely reduced the detection capabilities of the assay. The LOQ for the dilutions in ASW with contaminants was 0.625 µg/mL for all contaminant combinations tested, which falls well above the needed LOQ for diagnostic purposes. As such, another method of analyzing the specific protein quantity was evaluated.

3.4.1 Dipstick Assay

Like an ELISA, a lateral flow immunoassay (dipstick) relies on a “sandwich scheme” (Figure 4), with the specificity of monoclonal antibodies to different epitopes of the same antigen. However, the dipstick assay is much faster than the ELISA (~30 minutes versus at least 3 hours), and does not require a trained laboratory technician or specialized equipment (multichannel pipettor, plate reader) to complete. In the dipstick assay, one antibody is embedded on a nitrocellulose membrane, while the other is conjugated to gold particles. Typically, the top spot on the dipstick is the positive control for the assay, and underneath is the detection spot with an antibody specific for the target protein. The sample and gold-antibody conjugate are mixed, and wick up the membrane. The intensity of the target spot correlates with the protein levels present in the sample (Figure 4).

3.4.2 Dipstick detection of VtpA

We assembled lateral-flow dipsticks with the same antibodies that were used in the sandwich ELISA (12F8 for capture and 7C5 for detection). The capture antibody, 7C5, is spotted onto the dipstick, and binds the VtpA-12F8-collodial gold complex as it is wicked up the dipstick.

Based upon previous optimal antibody concentrations (Nadanaciva, Willis, Barker, Gharaibeh, Capaldi, Marusich and Will, 2009), 0.5 µg of monoclonal antibody 7C5 was spotted at 0.3 µL onto the lower 1/3 portion of the nitrocellulose membrane of each dipstick. Assays were later conducted to determine optimal dipstick antibody concentrations, testing 0.5, 0.75, 1.1, and 1.7 µg of 7C5/dipstick with increasing volumes of gold-conjugated 12F8 with low levels of *V. tubiashii* supernatant. Little to no increase in sensitivity or intensity of detection was noted (data not shown). The dipstick assay readily detected isolated His_6-VtpA (manufactured by GenScript, and also produced in-lab) with visual presentation of a positive colored detection spot (Figure 5). *V. tubiashii* (strain RE22) was grown in liquid culture and as expected (Hasegawa, Lind, Boin and Häse, 2008) high levels of protease were detected with the azocasein assay in the culture supernatants (results not shown). The dipsticks detected naturally-secreted protease of *V. tubiashii*, whereas a complete lack of signal was shown with the negative control (Figure 5).

3.4.3 Specificity of Detection of VtpA

In order to test the specificity of the dipstick assay for detection of the *V. tubiashii* protease, an additional strain of *V. tubiashii* (RE-98) was used to generate culture supernatants. Supernatant from two distantly-related *vibrios* (Thompson et al., 2006), *Vibrio splendidus* and *Vibrio mimicus*, were also tested. The dipstick assay showed clear detection of protease from supernatants of all tested *V. tubiashii* strains (Figure 6). However, no signal was detectable for *V. splendidus* and *V. mimicus* supernatants, or the negative control (Figure 6).

3.4.4 Sensitivity of Detection of VtpA

In addition to the specificity of the dipstick assay, the sensitivity of the assay for detection of low levels of VtpA protease was assayed. A 1:2 dilution series range of *V. tubiashii* supernatant (strain RE22) was prepared, ultimately testing as low as a 1:1024 dilution of the sample in buffer. Visible detection was noted down to a 1:64 dilution of the original bacterial supernatant (Figure 7). A dilution series was also performed with His_6-VtpA, and the dipstick was able to detect protease to 0.5 µg/mL (data not shown).

3.4.5 Effects of contaminants on the dipstick assay

Dipstick assay performance was tested in an environment of high contamination, as might be present in an ocean or hatchery environment. To that end, ASW was mixed with a 1:100 dilution of

phytoplex algae, and larvae were added in an approximately 1:10 dilution, giving the mixture a green tinge with numerous oyster larvae. This mixture of seawater, algae, and oyster larvae was then used in place of extraction buffer to create a *V. tubiashii* supernatant dilution series ranging from a 1:16 down to a 1:512 dilution. As a control, the same dilutions of protease-containing supernatant were performed in a background of extraction buffer which contained no environmental contaminants. There was little to no visible change in the detection ability of the assay under these highly contaminated conditions as compared to detection of *V. tubiashii*-secreted protease in control buffer (Figure 8).

3.4.6 Detection of Vibrio coralliilyticus metalloprotease

The secreted zinc-metalloprotease of *V. coralliilyticus* has been implicated in disease to coral reefs (Ben-Haim et al., 2003)and shares over 99% sequence homology of the mature protein to *V. tubiashii* VtpA. Given the sequence similarity of these two proteases, it was likely that our dipstick assay would also be able to detect the protease of the *V. coralliilyticus*. To test this, *V. coralliilyticus* and *V. tubiashii* supernatants were tested in an azocasein assay and were found to have similarly high levels of protease activity (data not shown). The same supernatant samples were then utilized in a 1:2 dilution series ranging from undiluted supernatant samples down to a 1:1024 dilution of sample. Detection of both the *V. tubiashii* and *V. coralliilyticus* protease was visible down to a 1:128 dilution of the original supernatant (Figure 9).

3.4.7 Positive control spot

To further improve this assay, work was next done to include a positive control on each dipstick. Although the wicking of solutions up the dipstick can be viewed during the incubation step, a negative result could be due to the absence of detectable protease, or to degradation of reagents and/or the gold colloid-antibody complex. The positive control spot is thus both a marker for the successful movement of liquid along the dipstick, as well as a positive signal that the reagents performed as expected. A positive control spot, composed of His_6-VtpA (GenScript Corp.) was added to dipsticks (Figure 10). The amount loaded per positive control spot was determined based on the optimal loading volume (0.3 µL) and concentration of the His_6-VtpA stock (data not shown).

4.1 DISCUSSION

The re-emergence of vibriosis in the Pacific Northwest in 2007 severely affected several regional shellfish hatcheries, and markedly reduced production (estimated decrease of 59%) at nursery and grow-out sites (Elston, Hasegawa, Humphrey, Polyak and Häse, 2008). For instance, Whiskey Creek Shellfish Hatchery located in Netarts Bay, OR (the largest commercial hatchery on the West Coast, supplying about 83% of seed for growers) reported dramatically reduced larval and juvenile production in recent years, resulting in near closure of the hatchery and economic hardships for many growers due to lack of seed for planting. Apart from the oyster hatcheries in the Pacific Northwest, unusually high oyster mortalities associated with *Vibrio* outbreaks have been recently reported in shellfish hatcheries in Florida, Hawaii, Mexico, Fiji, and Australia.

The ability to distinguish pathogens from non-pathogenic strains is a critically necessary tool for managing bacterial stability in shellfish hatcheries. Previous methods for detection of pathogenic *Vibrio* species, such as selective plating, bacterial agglutination, and protease activity assays, are relatively time-consuming (require 12-24 hours for optimal growth) as well as qualitative and potentially inaccurate and therefore cannot be effectively employed at production sites by producers. We recently developed a real time PCR assay, based on the *V. tubiashii* zinc-metalloprotease (*vtpA*) gene sequence, to not only monitor the presence of but also quantify *V. tubiashii* within environmental and hatchery seawater samples (Gharaibeh et al., 2009). Similar techniques have been successfully utilized for detection of *Vibrio alginolyticus* (Zhou et al., 2007) and *Vibrio vulnificus* (Panicker et al., 2004) within environmental seawater samples. Although qPCR is an accurate, highly sensitive method for absolute quantitative detection of bacterial levels, it is a laboratory method requiring complex equipment and trained technicians and thus is likely not suitable for application at production facilities. Moreover, it is important to note that the detection of the bacteria alone is not sufficient to effectively manage the larval mortalities associated with *V. tubiashii*, as the main toxin (the zinc-metalloprotease) of *V. tubiashii* is secreted by the bacteria and can kill oyster larvae in the absence of live bacterial cells (Hasegawa, Lind, Boin and Häse, 2008). At lower, more chronic exposure, the result may be slow larval growth, lack of metamorphosis and chronic mortality and morbidity, also effectively curtailing all useful production (Elston, Hasegawa, Humphrey, Polyak and Häse, 2008). Thus, there is a critical need for the development of sensitive detection methods for the accurate and quantitative detection of this key toxin.

The use of monoclonal antibodies raised against bacterial components has greatly expanded the ease of diagnostic testing, allowing for specific bacterial identification without the need for pre-culturing

or other enrichment processes. The ELISA (enzyme-linked immunoassay) allows for specific detection of the target compound, but this format relies on trained laboratory personnel and specialized equipment. The ELISA has been used for detection of *vibrios* (Honda et al., 1985, Kumar et al., 2011). A dipstick assay, in which the sample and detection antibodies are passively wicked over a membrane-bound capture antibody, requires substantially less time than an ELISA. This format has been successfully used for various diagnostic tests for *Vibrio cholerae* (Nato et al., 2003, Sithigorngul et al., 2007, Yu et al., 2011), and the detection capabilities posed by this format greatly expand the usefulness of an antibody-based assay.

In this paper, we have described the development of two specific tests for the *V. tubiashii* zinc-metalloprotease (VtpA). Both technologies rely on the specificity of monoclonal antibodies that recognize unique epitopes of the mature VtpA protein, and these antibodies both give colorimetric readouts. A sandwich ELISA was sensitive when VtpA was diluted into PBS, but was markedly less sensitive in conditions that correlate with the environment of hatchery-derived samples, such as in the presence of ASW, phytoplankton, or oyster larvae. In contrast, a dipstick assay proved to be equally as sensitive in the presence of contaminants, indicating that this rapid, sensitive and easy-to-use format would be the ideal assay method for detecting VtpA in a hatchery setting. A physiologically relevant detection range for this assay would allow for a detection of 0.1-1 µg/mL of VtpA (Hasegawa, Gharaibeh, Lind and Häse, 2009), however, this assay can currently detect a minimum of 0.5 ug/mL His_6-VtpA (data not shown). Further refinement of the dipstick assay will hopefully allow for increased detection sensitivity to increase its usefulness in the field. Additionally, a vibriosis event in the oyster hatchery setting would allow for the correlation of mortality data with the detection of VtpA by the dipstick assay.

In addition to the unusual losses in larval production experienced by the West Coast oyster farmers, there has been no commercial wild set of Pacific oysters in Willapa Bay, WA, over the last several years. It is also unknown to what degree larvae of native oysters, mussels and other shellfish were impacted in the past years and it is likely that *V. tubiashii* also has an adverse effect on larvae of other bivalve species under natural conditions. For example, Elston et al. (2008) isolated a pathogenic strain of *Vibrio* spp. associated with a precipitous loss of Olympia oyster (*Ostrea conchaphila*) larvae in Willapa Bay. Significant efforts are being made to restore Olympia oyster populations on the West Coast and clearly these efforts might be jeopardized by high mortalities due to *V. tubiashii.* A better understanding of the abundance and role of *V. tubaishii* and its toxin in natural settings are required. The detection assays described here could be useful tools for oyster restoration efforts, as these new technologies may allow more precise determination of concentrations of *V. tubiashii* and its toxin in

coastal waters than was previously possible. Significantly reduced populations of bivalves, a critical keystone species in the marine ecosystem, are bound to have significant environmental consequences. The reduction in water filtering performed by these shellfish will impact water quality, and diminished shellfish assemblages will reduce forage and refuge opportunities these three-dimensional structures normally provide for a host of marine flora and fauna.

The developed dipstick immunoassay does not detect secreted metalloproteases from both *V. splendidus* and *V. mimicus* (Figure 6). This is an indication of the specificity of the monoclonal antibodies used in the assay, as even zinc-metalloproteases secreted by related bacteria (Hasegawa, Lind, Boin and Häse, 2008) are not detected by the assay. The (zinc) metalloprotease of *V. tubiashii* and *V. splendidus* falls into Class I of the vibrio metalloproteases (HEXXH + E), while the metalloprotease of *V. mimicus* falls into Class II of vibrio metalloproteases (HEXXH)(Lee et al., 1998). Future work on the dipstick will expand the range of vibrio proteases tested as negative controls. *V. splendidus* affects a wide variety of aquaculture settings, including the corkwing wrasse (used with salmonid farming) (Jensen et al., 2003), carpet shell clams (Gomez-Leon et al., 2005), and turbot (Thomson et al., 2005). This has also been described as a pathogen of oyster larvae in Japan (Sugumar et al., 1998) and France (Lacoste et al., 2001). *V. mimicus* is a lesser problem in aquaculture. It has not been described to infect oysters, although it has been isolated from the hemolymph of crayfish (Wong et al., 1995).

In addition to detection of *V. tubiashii* VtpA, antibody conjugations capable of detecting diseases that plague hatchery settings may also be added to this dipstick format, providing a multiplexed diagnostic assay to facility managers. Salmonids are affected by *V. salmoncidia* (Egidius et al., 1986), while tiger prawn (*Penaeus modon)* are affected by *V. harveyi* , *V. alginolyticus* and *V. parahaemolyticus* (Lee et al., 1996, Liuxy et al., 1996, Sudheesh and Xu, 2001). Viral diseases also affect hatcheries, as ostreid herpesvirus (OsHV) causes summer mortality in oyster hatcheries in both Europe (Barbosa-Solomieu et al., 2005, Martenot et al., 2011, Renault et al., 1994) and the United States (Burge et al., 2007, Friedman et al., 2005).

Vibrio coralliilyticus is a known pathogen of corals and a very close relative of *V. tubiashii*. Interestingly, *V. coralliilyticus* secretes a zinc-metalloprotease that has been implicated in coral disease (Sussman et al., 2009). This enzyme is very similar to VtpA (99% similarity) and as coral disease diagnostic tools are urgently needed (Pollock et al., 2011), we assessed the capability of our dipstick assay to detect the secreted zinc-metalloproteases of *V. coralliilyticus*. As could be expected from the high similarity of these proteins, our dipstick assay readily recognized the secreted protease from *V. coralliilyticus*, thus broadening its potential usefulness to areas outside of the molluscan hatchery

setting. The ability to detect and quantify microbial agents identified as indicators of coral disease will aid in the elucidation of disease causation and facilitate coral disease diagnosis, pathogen monitoring in individuals and ecosystems, and identification of pathogen sources, vectors, and reservoirs. Overall, we anticipate providing the field of coral disease research as well as the commercial aquaculture industry with a useful tool to help them prevent bacterial diseases.

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Figure Legends

Figure 1. Direct ELISA for His_6-VtpA using 7C5. Protease was serially diluted in either PBS (blue diamonds) or ASW (red squares).

Figure 2. Sandwich ELISA for His_6-VtpA. VtpA-specific monoclonal antibody 12F8 was used to capture protease, which was serially diluted in either PBS (blue diamonds) or ASW (red squares).

Figure 3. Contaminants in sandwich ELISA format decrease detection sensitivity. His_6-VtpA was serially diluted in PBS, ASW, or ASW with various combinations of contaminants (larvae, algae, or both).

Figure 4. Lateral-flow dipstick immunoassay. When indicated, a positive control spot, protease alone, captures excess detection antibody. Image provided courtesy of Abcam Inc. Image ©2012 Abcam.

Figure 5. Detection of His_6-VtpA and VtpA in *V. tubiashii* supernatants by dipstick assay. A – GenScript His_6-VtpA; B – In-lab purified His_6-VtpA; C through E – *V. tubiashii* RE-22 supernatant; F – negative control.

Figure 6. Detection specificity of dipstick assay. A – RE-98 supernatant; B - RE-22 supernatant; C - *V. splendidus* supernatant; D - *V. mimicus* supernatant; E – negative control.

Figure 7. Sensitivity of the dipstick assay to *V. tubiashii* supernatant dilution. A dilution series was performed with RE-22 supernatant, and values below the images represent the dilution factor of the supernatant (4 refers to a 1:4 dilution). (-); negative control.

Figure 8. Dipstick assay testing *V. tubiashii* supernatant dilutions in extraction buffer (left), seawater + algae (middle), or seawater + oyster larvae + algae (right). Values above each dipstick refer to the dilution of *V. tubiashii* supernatant (16 refers to a 1:16 dilution). (-); negative control.

Figure 9. Testing of *V. tubiashii* RE-22 (left) and *V. coralliilyticus* (right) supernatants with dipstick immunoassay. Negative control is right-most strip on each. Values above each dipstick refer to the dilution of *V. tubiashii* supernatant (4 refers to a 1:4 dilution). (-); negative control.

Figure 10. Positive control detection with the dipstick assay. His_6-VtpA (top) or the VtpA specific monoclonal antibody 7C5 (bottom) were spotted onto the strip. *V. tubiashii* RE-22 supernatant was used on the left strip, no protease sample was used on the right strip (negative control).

Table 1. Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant Characteristics	Source/Reference
Vibrio tubiashii RE-22	Wild-type	Estes, Friedman et al. 2004
Vibrio tubiashii RE-98	Wild-type	Estes, Friedman et al. 2004
Vibrio splendidus LGP 32	Wild-type	Gay, Berthe et al. 2004
Vibrio mimicus ATCC 33653	Wild-type	Yi-Chen Su
Vibrio coralliilyticus ATCC BAA-450	Wild-type	ATCC
E. coli pBADvtpA-His	*vtpA* gene in pBAD-His	Hasegawa and Häse. 2009

Highlights

- Two detection methods have been developed for *Vibrio tubiashii* zinc metalloprotease
- The ELISA is significantly affected by contaminants, such as oyster larvae
- The dipstick immunoassay is not affected by contaminants, and is faster to run
- Aquaculture facilities could use the novel dipstick on-site for rapid testing for VtpA