Diagnosing Diseases of Concern to Hawaii Aquaculture Producers

RuthEllen Klinger-Bowen, Research Corporation University of Hawaii, Lei Yamasaki, Hawaii Department of Agriculture, Karin Kurkjian, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, and Jenee Odani, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa

Global food fish consumption has outpaced population growth by two to one according to the 2018 Food and Agriculture Organization (FAO) report on the state of world fisheries and aquaculture (FAO, 2018). It is projected that aquaculture production must grow 70% to feed the world population by 2050 (FAO, 2014). This goal cannot be met without anticipating the potential setbacks from economic and management issues. FAO firmly states that disease control is one of highest priorities to accommodate future aquaculture growth. The U.S. aquaculture industry alone loses $6 billion per year to disease issues (World Bank, 2014), ultimately constricting production.

The transportation of live animals is essential to all animal-producing sectors of agriculture. Animals are moved for a number of reasons, including marketing, restocking, slaughter and genetic program enhancement. Although movement is necessary for the growth and development of industries, it plays a significant role in the spread of infectious diseases. Because of this, many national and state governments have strict animal health import requirements to reduce the risk of disease introduction.

As the aquaculture industry in Hawaii grows, updated information on diseases is needed to protect investments and to meet requirements to move animals in and out of the state. For example, koi producers in Hawaii test their animals for koi herpesvirus (KHV) and spring viremia of carp (SVC) to meet the import requirements of countries. Koi producers will often import new stock and perform post-entry testing to confirm that the lot of fish are negative for both pathogens. Tilapia lake virus (TiLV) is an emerging viral disease of tilapia that has had worldwide implications for the aquaculture community (Fathi et al., 2017; Behera et al., 2018; Jansen et al., 2018). Testing for TiLV may eventually be required for any shipment of tilapia in and out of Hawaii. The bacterium *Francisella orientalis* (previously named *Francisella noatunensis* subsp. *orientalis* or Fno (Ramirez-Paredes et al., 2020)) is well known in Hawaii, particularly on Oahu (Soto et al., 2013). It continues to cause morbidities/mortalities in cultured tilapia during the winter months, which reduces revenue for the fish farmer. Another bacterium, *Streptococcus iniae*, is reported to infect 27 freshwater, marine, and estuarine species, including causing up to 50% mortality in tilapia (Agnew and Barnes, 2007). Ominously, this potentially zoonotic bacterium has been found in wild populations near aquaculture facilities in other parts of the world (Colorn et al., 2002). *Ostreid herpesvirus 1* (OsHV-1) is an emerging pathogen of oysters (OIE, 2019a), resulting in significant losses in Australia, New Zealand, and Europe (Whittington et al., 2018). A variant of OsHV-1 was reported for the first time in the U.S. in 2002.

Although the Hawaii Department of Agriculture regulates the import of live animals, seafood products that are frozen or fresh (on ice) enters Hawaii without being tested for diseases. Testing

marketed seafood would give the aquaculture industry an idea of what diseases are potentially introduced, and what they need to do to protect their investment.

To properly focus our project's objectives, we first determined the diseases of highest concern to Hawaii's aquaculture producers, via a survey that was sent via email and in person on four separate occasions. Based on the 13 responses, we also conducted a literature review to evaluate significance of the pathogens and our working group created a list of six pathogens that were determined to be of most concern: *F. orientalis*, TiLV, and *S. iniae* in tilapia, KHV and SVC in koi, and OsHV-1 in oysters.

Figure 1. Typical setup for fish tissue collection

In response to these concerns, twenty animals were submitted for testing from two koi, four tilapia, and five oyster facilities. Up to five animals were purchased from separate markets, including four cyprinid, five tilapia, and six oyster vendors. Tissues were selected for testing based on likelihood of being an indicator for the pathogen and to obtain a mix of lethal vs. non-lethal samples (Figures 1 & 2). Tissues collected from tilapia included spleen, liver, gill, fin, eye, and anterior kidney. Spleen, liver, gill, and fin were the primary target tissues; however, in two market cases where the fish were sold gutted, eye and anterior kidney were used as substitutes for spleen and liver (both *F. orientalis* and TiLV can cause ocular lesions, while anterior kidney can harbor *F. orientalis*). Tissues collected from cyprinids included spleen, posterior kidney, gill, and fin. Tissues collected from oysters included gill and mantle. No suitable non-lethal samples for OsHV were reported in the literature.

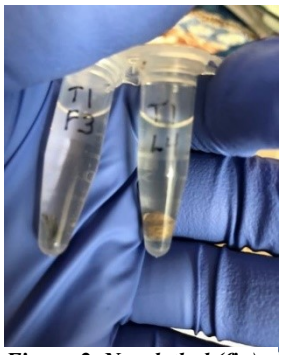

Figure 2. Non-lethal (fin) and lethal (liver) tissue in tubes with ethanol

After sample collection, tissues were sent to the University of Hawaii Animal Diagnostic Laboratory (UHADL). The Qiagen Kit DNeasy Blood & Tissue Kit and the RNeasy Mini Kit were used for DNA and RNA extraction, respectively, following the protocol suggested by the manufacturer. A real-time PCR (qPCR) assays for TiLV, *F. orientalis*, KHV, SVC, OsHV-1, and conventional PCR assay for *S. iniae* were developed following previously published methods (Waiyamitra et al. 2018; Gilad et al., 2004; OIE 2019a-c; Mata et al., 2004). The qPCR assays were conducted in duplicates and analyzed within the Applied Biosystems 7500 Fast Real-Time PCR Systems (Applied Biosystems). The GoTaq probe qPCR Master Mix from Promega was used for qPCR analysis and the GoTaq DNA Polymerase Kit was used for *S. iniae* samples (Figure 3).

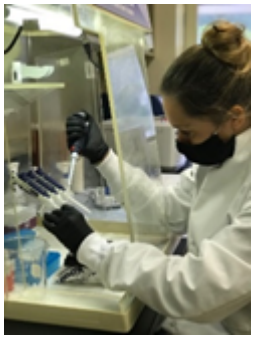

Figure 3. K. Kurkjian performing PCR assays

Of five tilapia farms tested, two had fish that were positive for *F. orientalis*. One farm had six of 20 fish test positive by PCR assay (spleen samples; 30% prevalence). The second farm had nine of 20 fish test positive by PCR assay (spleen samples; 45% prevalence). In the second farm, one of the nine fish had two tissues test positive by PCR (liver and spleen). These farms are located

on Oahu, an island known to host *F. orientalis* in aquacultured and wild tilapia populations (Yamasaki et al., 2020, Soto et al., 2013; Klinger et al., 2012; Tamaru et al., 2011). In total, 321 PCR assays for *F. orientalis* were completed. Cycle threshold (Ct) values and copy numbers for the 16 samples that tested positive for *F. orientalis* are presented in Table 2. Ct levels are inversely proportional to the amount of target nucleic acid in the sample: samples with lower Ct values have a higher amount of target nucleic acid or copy numbers.

Table 1. Number of positive results over the total number of samples tested from farms and markets. Some samples were not tested for all pathogens due to insufficient tissue.

Pathogen	Farmed	Market
KHV	0/160	0/100
SVC	0/160	0/100
TiLV	0/320	0/88
F. orientalis	16/233	0/88
S. iniae	0/233	0/88
OsHV-1	0/200	0/60
Total PCR assays	**1306**	**524**

Table 2. Threshold cycle and copy numbers per uL detected by qPCR for samples positive for F. orientalis.

Fish no.	Pathogen tested	Tissue sampled	Ct value	Copy number
1	*F. orientalis*	spleen	35.2849	8.79×10^3
2	*F. orientalis*	spleen	34.6542	1.30×10^4
3	*F. orientalis*	spleen	29.7687	2.71×10^5
4	*F. orientalis*	spleen	36.5711	4.04×10^3
5	*F. orientalis*	spleen	36.9348	3.22×10^3
6	*F. orientalis*	spleen	33.7747	2.55×10^4
7	*F. orientalis*	spleen	26.3255	5.47×10^6
8	*F. orientalis*	spleen	35.1091	2.45×10^4
9	*F. orientalis*	spleen	30.4051	4.78×10^5
10	*F. orientalis*	spleen	36.8216	1.12×10^4
11	*F. orientalis*	spleen	35.0141	2.79×10^4
12	*F. orientalis*	spleen	31.0548	1.40×10^6
13	*F. orientalis*	spleen	34.2588	2.53×10^5
14	*F. orientalis*	spleen	28.2528	5.79×10^6
15	*F. orientalis*	spleen	27.9435	7.05×10^6
15	*F. orientalis*	liver	37.5936	4.68×10^4

All non-lethal samples from the 15 tilapia that tested positive for *F. orientalis* using lethal samples (spleen +/- liver) tested negative. Only lethal samples collected for *F. orientalis* yielded positive results. The bacterium was not detected in any of the marketed tilapia, which were

frozen fish from China, Taiwan, and Thailand (Figure 4). TiLV and *S. iniae* were not detected in fish from farms or markets.

Figure 4. Farm-raised frozen tilapia from Taiwan

Both koi farms were negative for KHV and SVC. Live cyprinids (koi and feeder comets) were purchased from pet stores on Oahu; most were supplied from the mainland (California and Florida). One pet store received koi from Asia but the country of origin was unknown. Nevertheless, all the cyprinids were negative for the two pathogens.

None of the oyster farms were positive for OsHV-1. The oysters that were purchased in the markets were primarily shipped (live on ice) from northwest U.S.; however, there was one collection purchased from eastern U.S. and one (frozen) from Korea (Figure 5). None of the purchased oysters were positive for OsHV-1.

Figure 5. Frozen oysters from Korea

This project also examined non-lethal methods (gills, fins) to determine if valuable fish could be sampled without sacrifice in future health inspections. However, non-lethal samples (gills, fins) were not useful for detection of *F. orientalis* in tilapia and could not be evaluated for other pathogens since no positive animals were detected.

With the results of the survey, we were able to determine what aquatic animal diseases the aquaculture industry are concerned about. This led to the validation of PCR tests for TiLV, *F. orientalis*, KHV, SVC, OsHV-1, and *S. iniae*. The UHADL is now able to provide testing services to the industry for these pathogens. Imported seafood (tilapia, oysters) and pet fish (koi, comets) were found to be negative for the pathogens of concern. While continuous testing of imported aquacultured products would be the best way to monitor the market for disease introduction, this is not a cost-effective or time-efficient strategy. Based on our survey, one area that should be addressed is biosecurity of farm operations. The survey asked what biosecurity measures are in place on the farms. This included perimeter fencing/locked gates, allowing visitors, foot baths/wash stations, log books for visitor sign-ins, pest/animal control measures, treatment of incoming water, and quarantine for new animals. All had at least one biosecurity measure in place; however, some did not have basic measures such as quarantining new animals or visitor tracking.

Ideally, assessing the needs of Hawaii aquaculture producers should be conducted at least every five years to update primary concerns, report any new issues, and to gain insight on specific issues that researchers could address. The added benefit of establishing a local USDA approved diagnostic laboratory for Hawaii's aquaculture industry and potentially for other Pacific Islands creates a cost-effective and readily available service not only for the current pathogens but also for emerging aquatic diseases and biosecurity threats.

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