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Executive Summary

Megalocytiviruses are a group of closely related viruses that cause mass mortalities in both marine and freshwater aquaculture. They are of global importance as they are listed by the World Animal Health Organization (OIE). They are difficult to control as they infect over 125 fish species and individuals can be carriers for life with sporadic disease events resulting in considerable losses at farms. This project was developed by the University of Sydney following consultation with Department of Agriculture and Water Resources (DAWR), relevant state agencies dealing with aquatic biosecurity and from advice received from native fish and ornamental fish aquaculture producers. Research has shown that imported ornamental fish are a pathway for *Megalocytivirus* to enter Australia. There was a critical need to develop practical and efficacious disinfection protocols for recirculating aquaculture systems. We used ISKNV freshly amplified *in vivo* at low passage to evaluate eight disinfection procedures using a bioassay with Murray cod (*Maccullochella peelii*) as a sensitive test for infectious virus. The six disinfection protocols that produced negative bioassays and were considered effective are described in this report. Further, it was demonstrated that ISKNV remained infectious in aquarium water (void of fish) at 25°C for at least 48 hours. Recommendations have been made to revise operational and disease strategy manuals as part of AQUAVETPLAN and an awareness program for increased reporting of mortality events at ornamental fish aquatic facilities for emergency disease response. The research findings will be used by biosecurity regulators and farm managers for disinfection efficacy data specific for ISKNV. These virus specific data enable interpretation of disinfection guidelines for the prevention and control of disease caused by ISKNV. The project was funded by the Australian Government Department of Agriculture and Water Resources through the Fisheries Research and Development Corporation.

Background

The ornamental fish industry presents a high risk to Australia for introducing exotic pathogens and alien fish species with many documented occurrences. There have been several incursions of exotic aquatic pathogens of international significance. These include *Infectious spleen and kidney necrosis virus* (ISKNV) (Rimmer et al., 2015; Mohr et al., 2015), *Cyprinid herpesvirus 2* (Stephens et al., 2004; Becker et al., 2014), and *Edwardsiella ictaluri* (Humphrey et al., 1986, Kelly et al., 2018), with the latter two now considered endemic in wild fish populations. The megalocytiviruses, ISKNV and a closely related but distinct virus, Red sea bream iridovirus (RSIV) are notifiable to the OIE. ISKNV and RSIV are considered exotic to Australia and infection with these and related viruses is listed on the Australian Government Department of Agriculture and Water Resources national list of reportable diseases of aquatic animals.

This project was developed from the need to have disinfection protocols that are effective for ISKNV for the prevention and control of disease at recirculating aquaculture facilities. A range of practical methods were tested to ensure a suitable procedure could be adapted for the wide range of environments encountered in aquatic health. This project aimed to provide information needed to protect and support the domestic food and ornamental fish aquaculture industries and conservation and ecological sustainability of native fish populations.

Objective

The objective of this project was to identify effective disinfection measures to support biosecurity for ISKNV at aquaculture facilities.

Methodology

We freshly amplified ISKNV *in vivo* at low passage and prepared high titre samples in a matrix with defined biological load. Eight different disinfection procedures encompassing a range of chemical and physical procedures were evaluated and the efficacy was evaluated using a sensitive bioassay with Murray cod (*Maccullochella peelii*). Fish were injected with ISKNV samples immediately following the disinfection protocol and observed for up to 14 days. All treatments were tested in duplicate tanks at 23°C with 9 to 18 fish per tank. The bioassay was considered negative when all fish from a treatment group were qPCR negative for ISKNV. Appropriate positive and negative control tanks were run in parallel with the treatment groups. To test the stability of ISKNV in water, naïve Murray cod were added to an aquarium (at 25°C) previously containing fish with a clinical infection with the virus. All fish were tested by qPCR for the detection of ISKNV at the time of morbidity/mortality or at the end of the trial.

Results

Table 1 provides the summary results for all of the disinfection procedures tested and the bioassay outcomes. Effective disinfection for ISKNV under high soiling conditions was achieved using heating at 65°C for 20 minutes, chlorine at 1000 ppm, pH 3, pH 11, 1% Virkon™ and a quaternary ammonium. Our research approach used a diluted positive control to show the effect of a very low viral challenge and to allow for potential comparisons with suboptimal disinfection procedures. The diluted positive control group showed that the bioassay was more sensitive than the qPCR assay. We demonstrated that ISKNV was stable in aquarium water at 25°C for at least 48 hours after the removal of clinically-infected fish.

Implications for relevant stakeholders

Detailed disinfection protocols that are efficacious for ISKNV in field relevant sample matrices were developed and tested in this current project. Several practical methods were identified as being effective for disinfecting *Megalocytivirus*. Thus, suitable disinfection procedures can be selected and adapted for a wide range of aquatic facilities and equipment used in aquaculture. We need to improve our understanding of the pathobiology of ISKNV infection and disease. This requires research to identify the range of prevalence and virus load that impact the detection of *Megalocytivirus* by qPCR and how these relate to fish that pose a risk of biosecurity breach through release of infectious virus. Further studies are needed to determine host susceptibility for key aquaculture species (such as Australian strains of barramundi used in aquaculture) and to identify novel fish hosts. Recent literature describing recombination events between ISKNV and RSIV (Shiu et al., 2018) and ISKNV epidemics at barramundi sea cage farms in Vietnam (Dong et al., 2017) highlight the need to review existing biosecurity policy. The ISKNV risk pathway from the importation of marine ornamental fish and the potential for release in marine environments needs reconsideration to ensure Australia remains free of ISKNV and RSIV. Improvement in biosecurity could be achieved with an increase in surveillance activities aimed at improving reporting of mortality events for emergency disease response or voluntary specimen submission from moribund ornamental fish at wholesale and retail facilities.

Recommendations

1. Revision to the Operational Procedures Manual-Decontamination to include new knowledge generated from this project and other published literature.
2. Commission the drafting of an AQUAVETPLAN Disease Strategy Manual for infection with *Megalocytivirus*.
3. The ornamental fish industry stakeholders (e.g. importers, wholesale distribution centres, farms, retail outlets) need an awareness program to be informed of their responsibilities for emergency disease reporting.
4. Improve the awareness of the importance of biosecurity at aquatic facilities by targeting training for stakeholders in the ornamental fish industry.
5. There is an urgent need to conduct cross sectional surveys at the national level for the detection of ISKNV. Wide-scale surveys at pre-import premises, wholesale distribution hubs and retail outlets are urgently needed to compliment the voluntary testing of domestic breeders currently being funded by DAWR.

6. Develop and advance the validation of diagnostic tests that detect all members of the genus *Megalocytivirus*.

Keywords

biosecurity, disinfection, emergency disease response, exotic pathogen, *Megalocytivirus, Infectious spleen kidney and necrosis virus*, ISKNV, red bream iridovirus, RSIV, ornamental fish, Blue gourami, *Trichopodus trichopterus*, Dwarf gourami, *Trichogaster lalius*, Murray cod, *Maccullochella peelii,* barramundi, *Lates calcarifer*

Introduction

The ornamental fish industry presents a high risk to Australia for introducing exotic pathogens and alien fish species with many documented occurrences (Lintermans 2004; Padilla and Williams 2004; Rixon et al. 2005; Cohen et al. 2007; Cobo et al. 2010; Duggan 2010). Annually, Australia imports approximately 18 million ornamental fish predominantly from Indonesia, Singapore and Thailand (O'Sullivan et al., 2008). Australia is considered to have a highly regulated quarantine system with stringent controls compared to global standards for importing live fish (Whittington and Chong, 2007). However, despite these controls, there have been several incursions of exotic aquatic pathogens of international significance, including *Infectious spleen, and kidney necrosis virus* (ISKNV) (Rimmer et al., 2015), *Cyprinid herpesvirus 2* (Stephens et al., 2004; Becker et al., 2014), and *Edwardsiella ictaluri* (Humphrey et al., 1986, Kelly et al., 2018), with the latter two now considered endemic in wild fish populations. Based on an import risk analysis (Australian Department of Agriculture, 2014), from 1 March 2016, ornamental fish belonging to cichlid, gourami and poeciliid families will require health certification indicating freedom from infection with *Megalocytivirus*.

Megalocytivirus have a wide host range and have caused epidemics at marine and freshwater food fish aquaculture (Subramaniam et al., 2012). The megalocytiviruses, ISKNV and a closely related but distinct virus, Red sea bream iridovirus (RSIV) are notifiable to the World Organization for Animal Health (OIE, 2012) and are subject to regulation and notification in many countries (Hick et al., 2016). Prior research in our laboratories has shown that several Australian native fish, including Golden perch (*Macquaria ambigua*) and the endangered Macquarie perch (*Macquaria australasica*) are susceptible to infection with DGIV through cohabitation exposure at 23°C (Rimmer et al., 2017).

From previous projects funded by the FRDC (2009/044 and 2014/001), imported ornamental fish are a pathway for *Megalocytivirus* to enter Australia. ISKNV was detected in numerous domestic populations of ornamental fish at wholesalers and retailers and at one aquaculture facility (Rimmer et al., 2015). ISKNV is considered exotic to Australia and infection with this virus and related viruses is listed on the Australian Government, Department of Agriculture and Water Resources national list of reportable diseases of aquatic animals. Given the prior outbreak of ISKNV resulting in over 90% of Murray cod dying at a hatchery in Victoria, there is a real risk to domestic aquaculture if ISKNV was to become established. There was a critical need to develop and test biosecurity measures to manage the disease risk to Australian fish farms.

In the simplest of terms, biosecurity is the protection of living organisms from any type of infectious organism. It can be applied at a local level, such as at a single farm or a national or an international

level. One of the best ways to protect fish and safeguard industries and ecosystems is by preventing and controlling disease with a good biosecurity program. Effective disinfection procedures are important components of disease control and general guidelines for aquatic animal health are available from the OIE. However, interpretation and implementation by industry and regulatory authorities require pathogen-specific information.

General guidelines for disinfection in response to emergency aquatic animal diseases are provided in AquaVet Plan Operation Manual for Decontamination (Australian Department of Agriculture, Fisheries and Forestry, 2008) and by the OIE (2017). However, the survival of RSIV outside a host is unknown and disinfection with ether, formalin and chloroform are impractical, particularly without detailed application instructions (OIE 2012). Biosecurity regulators and farm managers require disinfection efficacy data specific for megalocytiviruses to interpret these guidelines for prevention and control of disease. Detailed disinfection protocols that are efficacious for ISKNV in field relevant sample matrices are required. A range of practical methods ensures a suitable procedure can be adapted for the wide range of environments encountered in aquatic health.

This project addressed research priorities identified by the FRDC through the annual competitive round for the Aquatic Animal Health and Biosecurity Subprogram. The objective of the project was to identify effective disinfection measures to support biosecurity for ISKNV at aquaculture facilities.

Objectives

Identify effective disinfection measures to support biosecurity for ISKNV at aquaculture facilities.

Method

Practical and efficacious disinfection protocols were identified for field relevant samples. Preparations of ISKNV in tissue samples provided authentic, complex biological matrices. Evaluation of the infectivity of samples before and after disinfection required a bioassay in the absence of a cell culture system. Precedents for such studies with large, enveloped DNA viruses are available using a marine mollusk model (Corbeil et al. 2012, Hick et al. 2016).

Fish and Experimental Conditions

Murray cod fingerlings were obtained from a commercial farm in New South Wales and held in aquaculture tanks (300 to 500 L) with constant aeration and biological filtration. Water temperature was held at 23 ± 1 °C through the use of an aquarium heater and recorded daily with a glass thermometer. Water changes were completed as needed to ensure nitrogenous wastes remained below 0.5 mg/L. pH was held between 7.0-7.2 and adjusted daily as needed using sodium bicarbonate. Fish were fed at 2% of the tank biomass once daily with crushed native fish sinking pellets (Skretting). Fish were acclimated to the 100 L Perspex experimental tanks for 48 hours prior to experimentation. Prior to handling and the intraperitoneal injection (IP), fish were anaesthetized by immersion in benzocaine (50–60 mg/L). Fish were euthanized as required with an overdose of benzocaine at 120 mg/L. Animal ethics approval was obtained from the University of Sydney Animal Ethics Committee (approval number: 2016/979).

Source of infective ISKNV

From archival stocks, the well-characterized ISKNV isolate DGIV_2010 (Rimmer et al., 2017) was amplified twice in Murray cod. This work was the essential first step as the evaluation of disinfectants requires virus that has not been subject to freeze-thaw cycles. The DGIV_2010 isolate was obtained

from a single dwarf gourami (*Trichogaster lalius*) obtained from an aquarium shop in Sydney, Australia that had a natural acquired subclinical infection. The disinfection trials were conducted on freshly amplified virus stock at *in-vivo* passage level three or four rather than a cryopreserved virus stock which was shown previously to have diminished infectivity (unpublished data).

Trial 1 (SVC 16/104): A group of 19 Murray cod (mean (±SD) total length 128 ± 8 mm) were intraperitoneally (IP) injected with 50 µl of archived ISKNV inoculum (1/10 of the preserved clarified tissue homogenate stored in 2010 from Rimmer et al., 2017). Fish were held in two aerated tanks (100 L habitable volume) at 23°C and maintained with an established canister biofilter. Fish were observed twice daily and euthanized in the event of morbidity or at 28 days and tested by qPCR for ISKNV. All qPCR positive fish had the kidney, liver and spleen tissues pooled, homogenized by bead beating and filtered at 0.22 µm to produce a 1/10 (w/v) tissue homogenate that was cryopreserved with 10% fetal bovine serum (FBS) and 10% glycerol at -80°C. This was used as the challenge inoculum in a second amplification trial (Trial 2). The mean copy number of this first passage *in vivo* virus stock was 9.74 x 10^8 per mg of fish tissue.

Trial 2 (SVC 16/143): Twenty Murray cod (total length 128 ± 8 mm) held in triplicate tanks at 25°C were injected with 50 µL of 1/10 dilution of the cryopreserved clarified tissue homogenate generated from Trial 1. The kidney, liver and spleen from these fish were pooled, homogenized by bead beating, filtered and cryopreserved as above. The clarified tissue homogenate inoculum from Trial 2 was used for the bioassays to generate fresh virus. The mean copy number was 7.43 x 10^5 per mg of fish tissue.

Disinfection treatments tested for ISKNV

For Trial 3 freshly amplified third passage virus was obtained by injecting 20 donor Murray cod with 50 µL of a 1/10 dilution of the cryopreserved ISKNV-infected clarified tissue homogenate generated from Trial 2. For Trial 4, 10 Murray cod were injected with 50 µL of a 1/10 dilution of the cryopreserved ISKNV-infected clarified tissue homogenate generated from Trial 3 (thus at 4^{th} passage).

The donor Murray cod were held at 25°C in aerated aquariums fitted with a canister biofilter. On Day 7, fish were euthanized and kidney, liver and spleen tissues were dissected and pooled. A tissue homogenate was prepared using a mortar and pestle with phosphate buffered saline (PBS) at 1/10 (w/v) and filtered at 0.22 µm. The clarified tissue homogenate was diluted at 1/10 and FBS (10% v/v) was added to the homogenate to create a standardized, authentic biological matrix. This was referred to as the "virus preparation" and was subjected to the disinfectant treatments as described below, and

kept on ice between steps. A qPCR was performed on clarified pooled tissue homogenate and the virus preparation.

Two positive controls were used in each bioassay with one being no treatment or buffer exchange only of the virus preparation. The second positive control preparation was a 1/100 dilution with PBS (w/v) of the virus preparation and was referred to as the diluted positive control. Effectively, the positive control and the diluted positive control groups were injected with 4.5 µg and 0.045 µg of ISKNV-infected tissue, respectively.

Negative control challenge inoculum was prepared using kidney, liver and spleen tissues from Golden perch, *Macquaria ambigua* (previously tested negative for MCV) that were homogenized with PBS (1:10 w/v) and then combined with 10% FBS (v/v).

The disinfection treatments as summarized in Table 1 were:

a) heating in a hybridization oven followed by rapid cooling on ice,

b) changes in pH by addition of HCl or NaOH and neutralized to pH 7, or

c) chemical treatment with Virkon™, chlorine (1000 ppm) or quaternary ammonium compound (Accent, 26 g/L benzalkonium chloride tested at 650 ppm).

Buffer exchange was performed for the chemical treatments to remove any residual disinfectants prior to injection. A regenerated cellulose 30 000 molecular weight cut-off membrane centrifuge device was prepared by pre-rinsing the membrane with ultrapure water. The buffer exchange device was loaded with the treated challenge inoculum and centrifuged at 4000 *g* for 5 minutes at 4°C (Hick et al., 2016). The same chemical and pH disinfection procedures were applied to the negative control tissue preparation. The quantity of ISKNV DNA was assessed by qPCR before and after each disinfection procedure. All of the challenge inoculums were held on ice until time of use and were injected into fish within 1 hour of preparation.

Bioassays

Two bioassay trials were completed with Trial 3 testing the effect of heating the tissue preparation (Table 2) and Trial 4 testing heat, pH and chemical disinfectants (Table 3). A bioassay outcome was considered negative when all fish from a treatment group were qPCR negative at the time of morbidity, at the 7 day post injection sampling and on the final day of observation.

Treatments and fish were randomly assigned to the experimental tanks (using www.random.org). Water quality was managed as described above and temperatures maintained at 23°C. All waste water was disinfected with >1000 ppm chlorine for 60 minutes prior to disposal. Fish were fed once daily five times per week to a maximum of 2% tank weight. Feeding was halted if fish did not show interest or feed was left remaining on tank bottom.

Disinfecting treatments were tested in duplicate tanks with 9 to 18 fish per tank. On Day 0, fish were given a 50 µL IP injection with the virus preparation that underwent a disinfecting treatment described above. Positive controls were run in duplicate tanks with six to 20 fish in a tank and negative controls were run in single tanks. Fish were monitored twice daily for signs of moribidity. Moribund or dead fish were collected and frozen whole at -80°C for qPCR testing. On Day 7, one third of the fish were randomly selected, euthanized and frozen at -80°C for qPCR testing. All surviving fish were sampled on Day 14 for Trial 3 and Day 12 for Trial 4.

Stability of ISKNV in water

To test the stability of the ISKNV in an appropriate biological matrix, naïve Murray cod were added to aquariums previously containing cod with a clinical infection with the virus (Trial 5). Once the infected fish were removed, the aquarium was maintained at 25°C with the same biofilter and without water change. The naïve fish were added to tanks 1 h, 24 h or 48 h following the removal of the infected fish. Fish were fed as above and monitored daily for signs of morbidity. All fish were euthanized and tested for ISKNV by qPCR on Day 14.

Quantitative PCR testing for ISKNV

Tissue preparation, nucleic acid purification and qPCR testing was completed as described previously in Rimmer et al. (2012) and Becker et al. (2017). The MCV assay uses SYBR Green detection chemistry to target a conserved region of the major capsid protein (MCP) gene. Briefly, kidney, liver and spleen were aseptically dissected from each fish. A 1/10 clarified tissue homogenate was produced by bead beating (TissueLyser II, Qiagen). Total nucleic acids were purified from 50 µL of the clarified tissue homogenate using the MaxMax-96 Viral Isolation kit (Ambion) in a MaxMax Express-96 magnetic particle processor (Applied Biosystems) according to the manufacturer's directions. The extracted DNA samples were held at -80°C. All samples were tested in duplicate reactions prepared with 5 µL of undiluted nucleic acid template in a total reaction volume equal to 25 µL. Thermocycling was according

to the following protocol: 95°C for 15min (hot start activation); 40 cycles of 95°C for 30s (denaturation), 62°C for 30s (annealing), 72°C for 30s (extension); followed by a dissociation curve. Samples with a Ct value were considered positive only if the Tm was in the range 84 – 86°C. The positive control for this assay was a dilution of total nucleic acids from a fish infected with the previously characterized ISKNV isolate, DGIV_2010 (Rimmer et al., 2012). The plasmid standard pDGIV-MCP1, which contained a 694 nucleotide portion of MCP gene sequence, was used for quantification (Rimmer et al., 2012).

Results, Discussion and Conclusions

Trial 1 and Trial 2

From Trial 1, 9 of 19 Murray cod died and were qPCR positive. The peak mortality period was between Day 20 and 25 post injection. A further 3 survivors at Day 28 were positive for ISKNV. Target tissues from all 12 positive fish were pooled and the mean copy number was 9.74×10^8 per mg of fish tissue. From Trial 2, all 20 Murray cod injected with ISKNV-infected material (from Trial 1) died by Day 7. All fish were qPCR positive and the mean copy number for the pooled clarified tissue homogenate was 7.43×10^5 DNA copies per mg of fish tissue. The ISKNV-infected material generated from Trial 2 was used to inject the donor Murray cod to generate the material evaluated for disinfection using bioassays.

Donor Murray cod for Trial 3 and Trial 4

For Trial 3, target tissues were pooled from 12 Murray cod that were euthanized on Day 7 post injection. The mean copy number for the pooled tissue homogenate (1/10) was 3.62×10^6 per mL. The mean copy number for the virus preparation prior to disinfection was 5.7×10^5 per mL. Therefore, the amount of ISKNV in the injection dose (50 µL) for the positive control fish was 2.81×10^4 copies. The remaining eight Murray cod were euthanized on Day 16, whereby most fish were showing signs of morbidity. The target tissues were pooled and cryopreserved as a clarified tissue homogenate for use in Trial 4. The mean copy number for the clarified tissue homogenate was 1.3×10^6 per mg of fish tissue.

Similarly, for Trial 4, target tissues were pooled from 10 Murray cod that were euthanized on Day 7 post injection. The mean copy number for the virus preparation prior to the buffer exchange procedure was 5.29×10^6 copies per mL and after buffer exchange was 3.2×10^6 copies per mL for the positive control. Therefore, the amount of ISKNV DNA in the injection dose for the positive control with buffer exchange was 1.6×10^5 copies.

Bioassays

An overall summary of the disinfection procedures tested and the bioassay outcomes was provided in Table 1.

Heating

Heating the virus preparation in the sample matrix to less than 60°C was not effective to inactivate ISKNV, with both bioassays being positive (Table 2). However, heating at 65°C for 20 minutes was effective despite the detection of viral DNA by qPCR in the inoculum after treatment (Table 3). The detected ISKNV DNA was not infectious as none of the 17 fish injected amplified any virus at 7 or 14 days (Table 3).

Chemical disinfectants and pH disruption

From Trial 4, all of the chemicals tested at the stated concentration and contract time were effective to disinfect ISKNV. Further, adjusting the pH to 3 and 11 was effective to disinfect ISKNV. The challenge inoculum for both the pH groups and the quaternary ammonia compound retained viral DNA that could be detected by qPCR, but it was non-functional as there was no *in vivo* amplification (Table 3). There was no detectable viral DNA in the challenge inoculum after the chlorine or Virkon™ treatments.

Positive Control Groups

All of the positive control groups were positive for their bioassay outcome (Table 1). Clinically-affected Murray cod were observed in the positive control groups for Trials 3 and 4. From Trial 3, 7 fish died between Day 3 to 7 with 4 of these being ISKNV positive (Table 2). All of the fish sampled on the final observation day (Day 14) were positive with a median copy number of 3.2×10^7 per mg of tissue (Table 2). Similar results were observed in Trial 4 with all 12 Murray cod being positive for ISKNV by Day 14 (Table 3). The median copy number was between 3.8×10^7 and 4.7×10^8 (Table 3).

The diluted positive control groups were used to show the effects of a low viral challenge and to allow for potential comparisons with suboptimal disinfection procedures. The diluted positive control groups demonstrated that the bioassay was more sensitive than the qPCR assay. Despite having an initial injection dose below the level of quantification, the diluted positive control resulted in a positive bioassay (Table 2 and 3). Trial 3 results showed that 90% of the Murray cod injected with the diluted positive control were positive with a median copy number of 6.3×10^4 (Table 2). From Trial 4, 10 of 18 fish injected with the diluted positive control were positive for ISKNV by Day 14 with a median copy number greater than 2.5×10^5 (Table 3).

Negative Controls

All fish in the negative control tanks were negative by qPCR at the end of the observation period for both Trial 3 (Table 2) and Trial 4 (Table 4). One fish died in Trial 4 and it was attributed to non-specific cause.

Trial 5 Stability of ISKNV in water

ISKNV was able to remain infective in aquarium water alone (i.e. void of fish) for at least 48 hours (Table 5). From Trial 5, infection occurred when naïve Murray cod were added to aquariums at 1, 24 and 48 hours after the last ISKNV-infected fish were removed. When added at 1 h, all of the fish died between Day 4 and 7 post exposure. All fish were positive for ISKNV and had a median copy number of at least 6.8×10^5 (Table 5). When fish were added after 24 or 48 h, we observed 1/6 (16.7%) were infected with ISKNV in all replicate tanks (Table 5). Two fish added after 48 hours died on Day 4 with clinical signs and both had very high copy numbers ($2.7 - 4.0 \times 10^8$ copies per mg).

Disinfection of Megalocytiviruses

For this project, the challenge inoculum used to test disinfection procedures was freshly harvested ISKNV-infected tissues that were partially purified and standardized with a biological matrix. We added 10% foetal bovine serum to ensure the disinfectants were being tested under a high level of soiling as set out in the guidelines for data to support the efficacy of disinfectants for veterinary use from the APVMA website (https://apvma.gov.au/node/1026) and the ASTM International standard (ASTM 2018). The high soiling conditions would be particularly relevant under circumstances of disinfection in an emergency response to an exotic disease outbreak. Further, the high soiling conditions are relevant to disinfectants that are inactivated by organic matter (such as chlorine). This project demonstrated effective disinfection could be achieved for ISKNV under high soiling conditions using heating at 65°C, chlorine, pH 3, pH 11, Virkon™ and quaternary ammonium compound at the tested concentrations and contact times shown in Table 1.

Previous research has shown ISKNV to be susceptible to chemical treatments and heating for disinfection. Chemical disinfection was shown effective using formaldehyde (2000 ppm), potassium permanganate (100 ppm) and sodium hypochlorite (200 ppm) with 15 minutes of contact time using ISKNV-infected spleen and kidney tissue filtrates in PBS (i.e. low soiling conditions) (He et al., 2002). Further, it was shown that heating at 50°C for 30 minutes and applying a pH of 11 for 30 minutes at 30°C was able to disinfect ISKNV filtrates (He et al., 2002). However, using the same testing conditions, iodine and pH 3 for 30 minutes at 30°C were ineffective at disinfecting ISKNV filtrates (He et al., 2002).

Using cell culture (i.e. low soiling conditions), RSIV has been shown to be sensitive to pH 3 and heat treatment at 56°C for 30 minutes (Kurita and Nakajima, 2012, Nakajima and Sorimachi, 1994). Similar to ISKNV, *Epizootic hematopoietic necrosis virus* (EHNV) is a large DNA virus belonging to the Family *Iridoviridae*. Using cell culture testing methods, Langdon (1989) reported that EHNV was non-functional following exposure to pH 4 or 12 for 1 hour, chlorine at 200 ppm for 2 h and heating to 60°C for 15 minutes or to 40°C for 24 hours. Changes to increase the soiling conditions in the above studies may affect the efficacy of the disinfection procedures.

Transmission of Megalocytiviruses

This project demonstrated that ISKNV remained infectious in aquarium water at 25°C for at least 48 hours after the removal of clinically-infected fish. There is no known information regarding the persistence of RSIV outside the host (OIE, 2012). Further research is needed to determine the window of infectivity for ISKNV-contaminated water.

Megalocytiviruses including ISKNV and RSIV widely infect marine and freshwater fish species in Southeast Asia with the host boundary between the two viruses being considered unclear (Kurita and Nakajima, 2012). Dong et al., (2017) confirmed natural epidemics of ISKNV infections in barramundi (*Lates calcarifer*) in marine farms from Vietnam. Between 2012 and 2014, annual outbreaks of ISKNV infections resulted in 40% to 46% mortality with younger barramundi (e.g. 3 to 15 g) being most susceptible (Dong et al., 2017). ISKNV distributes systemically via the circulatory system and ISKNV-infected cells have been identified in hematopoietic organs and most tissues, including muscle, skin and eyes (Xu et al., 2008, 2010). The importation of frozen aquaculture products with ISKNV-infected tissues could present a biosecurity risk to Australia. Recently, it was shown that RSIV-Ku isolated from diseased red seabream (*Pagrus major*) in Taiwan was a natural recombinant megalocytivirus of ISKNV and RSIV (Shiu et al., 2018). Given the opportunity for co-infection with ISKNV and RSIV, recombination events are possible. Continued research is needed to study the evolution and host range of megalocytiviruses.

Conclusions

Detailed disinfection protocols that are efficacious for ISKNV in field relevant sample matrices were developed and tested. Several practical methods were identified as being effective for disinfecting *Megalocytivirus*.

In summary, the effective disinfection procedures for ISKNV were:

- heating to 65°C for 20 minutes
- exposure to pH 3 for 30 minutes
- exposure to pH 11 for 30 minutes
- Virkon™ at 1% for 10 minutes
- Chlorine at 1000 ppm for 30 minutes
- Quaternary ammonium compound (0.2% Benzalkonium chloride) at 650 ppm for 10 minutes

Table 1. Summary of disinfection treatments tested for inactivating ISKNV. The challenge inoculum was freshly amplified, high titre ISKNV that was partially purified from fish tissue and added to a standardized biological matrix with 10% fetal bovine serum (v/v).

Treatment (Trial number)	Contact Time (minutes)	Outcome	
		qPCR assay following treatment[1,2]	Bioassay
Disinfection treatment			
Heating to 40°C	20	5.58×10^3	Positive
Heating to 60°C	5	9.58×10^3	Positive
Heating to 65°C	20	3.5×10^4	Negative
pH 3	30	7.4×10^4	Negative
pH 11	30	2.0×10^4	Negative
Virkon™ 1%	10	0	Negative
Chlorine at 1000 ppm	30	0	Negative
Quaternary ammonium compound at 650 ppm	10	5.0×10^4	Negative
Positive Controls			
No treatment (3)	0	2.81×10^4	Positive
1/100 dilution with PBS (3)	0	BLOQ	Positive
Buffer exchange (4)	0	1.6×10^5	Positive
1/100 dilution with PBS (4)	0	BLOQ	Positive
Negative Controls			
No treatment (3)	0	0	Negative
pH 3	30	0	Negative
pH 11	30	0	Negative
Virkon™ 1%	10	0	Negative
Chlorine at 1000 ppm	30	0	Negative
Quaternary ammonium compound at 650 ppm	10	0	Negative

[1] ISKNV genome copies per injection

[2] Below the level of quantification (BLOQ) from an inconclusive qPCR result from one of the duplicate reactions showing a high Ct result.

Table 2. Trial 3: The disinfection of ISKNV using heat as assessed by bioassay and qPCR.
The challenge inoculum was freshly amplified, high titre ISKNV that was partially purified from Murray cod tissue and added to a standardized biological matrix of 10% fetal bovine serum (v/v).

Treatment	Amount of ISKNV in injection[1]	n	Days post injection	No. of moribund fish	No. positive/ total no. tested	Median virus copy number (per mg of tissue)[2]	Bioassay outcome
40°C for 20 min	5.58×10^3	36	1-6	3	1/3	1.8×10^2	Positive
			7	0	9/11	3.4×10^4	Positive
			14	0	21/22	9.5×10^4	Positive
60°C for 5 min	9.58×10^3	36	4	1	1/1	7.2×10^2	Positive
			7	0	11/12	1.4×10^4	Positive
			14	0	23/23	9.4×10^5	Positive
Positive control	2.81×10^4	37	3-7	7	4/7	2.8×10^4	Positive
			14	0	30/30	3.2×10^7	Positive
Diluted positive control	BLOQ[3]	30	14	0	27/30	6.3×10^4	Positive
Negative control	0	30	14	0	0/30		Negative

[1] Virus preparation was tested by qPCR following treatment.
[2] Statistic only included qPCR positive fish.
[3] Below the level of quantification (BLOQ) from an inconclusive qPCR results from one of two wells tested showing a high Ct results

Table 3. Trial 4: The disinfection of ISKNV as assessed by bioassay and qPCR.
The challenge inoculum was freshly amplified, high titre ISKNV that was partially purified from Murray cod tissue and added to a standardized biological matrix with 10% fetal bovine serum (v/v).

Treatment	Replicate	Amount of ISKNV in injection[1,2]	Proportion of moribund fish	No. positive/ total no. tested	Median virus copy number (per mg of tissue)[3]	Bioassay outcome
Positive control[4]	1	1.6 x 10^5	5/6	6/6	3.8 x 10^7	Positive
	2		3/6	6/6	4.7 x 10^8	Positive
Diluted positive control[5]	1	BLOQ	0/9	3/9	2.5 x 10^5	Positive
	2		1/9	7/9	3.5 x 10^7	Positive
Chlorine 1000 ppm for 30 min	1	0	1/9	0/9	0	Negative
	2		7/9	0/9	0	Negative
65°C for 20 min	1	3.5 x 10^4	0/8	0/8	0	Negative
	2		4/9	0/9	0	Negative
pH 3 for 30 min	1	7.4 x 10^4	2/8	0/8	0	Negative
	2		0/9	0/9	0	Negative
pH 11 for 30 min	1	2.0 x 10^4	4/9	0/9	0	Negative
	2		0/9	0/9	0	Negative
Quaternary ammonium compound 650 ppm for 10 min	1	5.0 x 10^4	3/9	0/9	0	Negative
	2		3/10	0/10	0	Negative
Virkon™ 1% for 10 min	1	0	6/9	0/9	0	Negative
	2		1/8	0/8	0	Negative

[1] Virus preparation was tested by qPCR following treatment
[2] Below the level of quantification (BLOQ) from an inconclusive qPCR result from one of two wells tested showing a high Ct result
[3] Statistic only included qPCR positive fish
[4] Virus preparation was subjected to the buffer exchange procedure and then tested by qPCR
[5] Virus preparation was not subjected to the butter exchange procedure prior to being tested by qPCR

Table 4. Trial 4: The negative control groups tested with ISKNV-negative challenge material.

Treatment[1]	Amount of ISKNV in injection[2]	n	Proportion of moribund fish	Days post injection	No. positive/ total no tested	Virus copy number (per mg of tissue)	Bioassay outcome
Chlorine	0	6	0/6	14	0/6	0	Negative
pH 3	0	5	0/5	14	0/5	0	Negative
pH 11	0	6	0/6	14	0/6	0	Negative
Quaternary ammonium compound	0	6	1/6	14	0/6	0	Negative
Virkon™	0	6	0/6	14	0/6	0	Negative

[1] Treatment time and concentration was the same indicated in Table 3.

[2] Challenge inoculum was tested by qPCR following treatment.

Table 5. Trial 5: Stability of ISKNV in water as assessed by bioassay and qPCR.
Naïve Murray cod were added to an aquarium previously holding fish with clinical disease after 1 hour, 24 hours, or 48 hours from the infected fish being removed.

Description of aquarium prior to naïve fish being added[1]	Time added (h)	Replicate	n	Proportion of fish died	No. positive/ total no tested	Median of virus copy number per mg of tissue[2]	Bioassay outcome
6 Murray cod with clinical disease and high copy number	1	1	6	6/6	6/6	6.8×10^5	Positive
		2	6	6/6	6/6	1.7×10^6	Positive
5 Murray cod with clinical disease and high copy number	24	1	6	0/6	1/6	8.6×10^2	Positive
6 Murray cod with clinical disease and high copy number	48	1	6	1/6	1/6	2.7×10^8	Positive
		2	6	1/6	1/6	4.0×10^8	Positive

[1] aquarium (100 L) was held at 25°C with the existing biological filterer and no water change during the time the tank was void of fish
[2] Statistic only included qPCR positive fish.

Implications

Biosecurity risk mitigation needed for *Megalocytivirus*

General guidelines for disinfection in response to emergency aquatic animal diseases are provided in the Operational Procedures Manual — Decontamination (Australian Department of Agriculture, Fisheries and Forestry, 2008) and in Chapter 4.3 of the OIE Aquatic Animal Health Code (OIE, 2017). Biosecurity regulators and farm managers require disinfection efficacy data specific for megalocytiviruses to interpret these guidelines for prevention and control of disease. Detailed disinfection protocols that are efficacious for ISKNV in field relevant sample matrices were developed and tested in this current project. Several practical methods were identified as being effective for disinfecting *Megalocytivirus*. Thus, a suitable disinfection procedure can be selected and adapted for the wide range of aquatic facilities and equipment used in aquaculture.

Improved understanding of the pathobiology of ISKNV infection and disease requires research to identify the range of prevalence and virus load that impact the detection of *Megalocytivirus* by qPCR and how these relate to fish that pose a risk of biosecurity breach through release of infectious virus. Improvement in biosecurity could be achieved with an increase in surveillance activities aimed at improving voluntary specimen submissions of moribund ornamental fish at wholesale and retail facilities. Further knowledge is needed about the release and risk pathways for MCV within Australia. Recognising that an infectious dose of ISKNV can be below the limit of detection of qPCR, there is a need for characterisation of available assays through accepted validation pathways. This knowledge will be used to inform a risk-based surveillance system, which can inform an increase in the minimum expected prevalence for which surveillance needs to be targeted.

Recommendations

1. Revision to the Operational Procedures Manual – Decontamination is needed as the current version was written in 2008. The manual should be revised to include the new knowledge generated from this project and other published literature since the inception of the manual a decade ago.

2. Commission the drafting of an AQUAVETPLAN Disease Strategy Manual for infection with *Megalocytivirus*, with particular reference to ISKNV and RSIV. The disinfection protocols from this current project should be adopted as part of the emergency disease response plan for this virus family.

3. The importation of ornamental fish presents a high risk for the introduction of exotic pathogens to Australia. This has been documented with ISKNV, Cyprinid herpesvirus 2, and *Edwardsiella ictaluri*. The ornamental fish industry stakeholders (e.g. importers, wholesale distribution centres, farms, retail outlets) need an awareness program to be informed of their responsibilities for emergency disease reporting. From this project, ISKNV was shown to be infectious in aquarium water void of fish for at least 48 hours. There is a high risk of transmission of ISKNV with subclinical fish being sold to the public, moved to another retail outlet and potentially released in the natural environment. The wholesale distributors and retail outlets are considered a heterogeneous mixture of populations of ornamental fish. Consultation with industry stakeholders is needed to define criteria to identify trigger points for mortality events and reporting requirements to ensure an emergency disease response can follow. The disinfection protocols developed in this project could be used to decontaminate infected water and systems to prevent further spread of the virus. Improvements in reporting of mortality events would be an effective surveillance tool to detect other emerging and unknown exotic pathogens with other diagnostic tools (e.g. histopathology) as part of emergency disease response.

4. There is a need to improve the awareness of the importance of biosecurity at aquatic facilities with respect to ISKNV (and other diseases). Further, there is a need to provide training at an industry-level for developing and implementing biosecurity plans that target the ornamental fish industry. It is recommended to hold industry-focussed days for stakeholders in the ornamental fish industry to learn and practice the disinfection procedures.

5. There is a need for further research on pathogenesis of ISKNV infection including duration of persistent infections and potential for recrudescence of disease or shedding of virus under different water temperature and stress scenarios. Further host susceptibility studies are needed to identify risks to key aquaculture species in Australia, such as barramundi.

Further development

1. From FRDC 2014/001, the importation of marine ornamental fish is a pathway for introduction of *Megalocytivirus* (e.g. RSIV and ISKNV) into Australia. There is a need for a cross sectional survey to test imported freshwater and marine ornamental fish for subclinical infections with *Megalocytivirus* and to distinguish between RSIV and ISKNV.

   Concurrent cross sectional surveys at the national level for the detection of ISKNV are needed across the domestic ornamental fish supply chain. DAWR is implementing a survey of farmed ornamental fish to test for ISKNV with voluntary submissions by farmers and hobbyists. Separate surveys are needed to target ornamental fish populations at (1) pre-import premises, (2) wholesale distribution hubs and at (3) retail outlets. All surveys need to run in parallel to have a national "snap shot" for determining the prevalence of ISKNV in ornamental fish populations. This data will strengthen Australia's position for the existing import conditions if challenged by exporting countries. This will support the revision of biosecurity policy to prevent incursions of exotic megalocytiviruses. Further, it would be ideal to test privately owned sick/moribund/dead ornamental fish.

2. There is a need to quantify the release pathways for ornamental fish infected with pathogens of biosecurity significance in Australia. In particular, there is a need to assess the human-assisted release pathways for ornamental fish into natural waterways (marine and freshwater). This knowledge will support the proposed changes to the way that the DAWR manages the disease risks associated with imported ornamental fish. These data will be applicable to informing biosecurity risks for any exotic ornamental fish pathogen.

3. There is a need for ongoing validation of diagnostic procedures suitable for surveillance for all members of the *Megalocytivirus* genus. qPCR should be evaluated in a relevant range of host species and for all 3 known MCV genotypes including Turbot reddish body iridovirus (TRBIV) (Go et al 2016). Such data needs to be incorporated as recommended qPCR protocols in the OIE Manual of Diagnostic Tests for Aquatic Animals.

4. There is a need to continue research in the pathobiology of *Megalocytivirus* to better inform biosecurity risks to Australia. Ornamental fish are known to be subclinical carriers of MCV (Becker et al., 2017; Rimmer et al., 2015). There is limited information available regarding the pathogenesis of ISKNV in subclinically-infected fish and under what host and environmental conditions they can shed live virus and infect other individuals. Practical, industry-relevant environmental factors (e.g. sudden changes in temperature, high nitrogen conditions, organic loading, crowding) need to be evaluated under controlled conditions. Trials should simulate realistic conditions that ornamental fish are likely to encounter throughout the supply chain (e.g. long and short haul transportation, wholesale and retail holding facilities).

Extension and Adoption

The project findings will support emergency disease outbreak measures and will have direct benefit to many stakeholders. The findings identified sufficient disinfection protocols for ISKNV in recirculating aquaculture systems. Extension of the key outcomes will be ongoing through consultation with the Australian Department of Agriculture and Water Resources.

During the project, extension of the key findings to government and industry stakeholders was achieved by conference presentations and informal discussions at conferences. At the end of the project, there was a formal meeting on 22 February 2018 with key stakeholders at DAWR listed below.

a) Animal Biosecurity – Aquatics: (Helen Walker, Peter Stoutjesdijk, David Crass)
b) Animal Health Policy Branch – Aquatic Pest (Yuko Hood)

The topics covered in the meeting included discussions on research outcomes from current and past projects funded by FRDC on ISKNV, recommendations and policy implications from FRDC 014/001, future research avenues under current regulatory constraints

Project coverage

None.

Project materials developed

Conference Presentations

Becker JA. Risks to Australia's biosecurity from the trade of ornamental fish. UNSW Aquatics Symposium, 31 Jan- 2 Feb 2018, Sydney, Australia

Becker JA, Hick P, Trujillo Gonzalez A, Miller T, Hutson K. Risks to Australia's biosecurity from the trade of ornamental fish. 8th International Aquatic Animal Health Symposium, Charlottetown, Canada, 2-6 September 2018.

Becker JA, Hick PM, Hutson KS, Trujillo Gonzalez A, Tweedie A, Miller TL, Whittington R. and Robinson A. Strategic approaches to identifying pathogens of imported ornamental fish and the risk posed to biosecurity in Australia. 4th Australasian Scientific Conference on Aquatic Animal Health, Cairns, Australia, July, 2017.

Fusianto C. Applications of epidemiology and molecular technology for improved management of *Megalocytivirus* infections in aquaculture. Sydney School of Veterinary Science Post Graduate Conference, 7 November 2017, Sydney, Australia

Fusianto C, Hick P, Becker J. Disinfection measures for *Megalocytivirus* at aquaculture facilities. UNSW Aquatics Symposium, 31 Jan- 2 Feb 2018, Sydney, Australia

Fusianto C, Hick P, Becker J. Effective disinfection protocols for Megalocytiviruses. 8th International Aquatic Animal Health Symposium, Charlottetown, Canada, 2-6 September 2018.

Publications

Fusianto C, Hick PM, Becker JA. Stability of *Infectious spleen and kidney necrosis virus* and susceptibility to physical and chemical disinfectants. *Aquaculture* (under revision as of 14 January 2019).

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Appendices

Appendix 1: List of researchers and project staff

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Appendix 2: Intellectual Property

This project has not developed any intellectual property that requires legal protection.