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The impact of pooling samples on surveillance sensitivity for the megalocytivirus *Infectious spleen and kidney necrosis virus*

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Summary

Movements of large volumes and species varieties makes the ornamental fish industry a high-risk pathway for the transfer of aquatic pathogens to new geographical regions and naïve hosts, potentially resulting in emergency disease events. *Infectious spleen, and kidney necrosis virus* (genus *Megalocyttivirus*) is considered exotic to Australia despite documented incursions since 2003. There are current import controls requiring freedom from infection for entry to Australia. The objective was to evaluate the effect of tissue pooling strategies for qPCR testing using a SYBR® assay for freedom from ISKNV at 2% expected prevalence with 95% confidence. Tissue homogenates from apparently healthy imported ornamental fish were tested as individuals and in pools of 5 and 10. Analytical sensitivity of the qPCR assay was reduced by two orders of magnitude when the nucleic acid extraction process was accounted for by spiking the plasmid in fish tissues and compared to molecular grade water. Diagnostic sensitivity of the assay was substantially reduced when testing tissues in pools compared to individual testing. For Population 1 (66% positive for ISKNV with moderate viral loads), surveillance sensitivity was only achieved using individual testing. For Population 2 (100% positive ISKNV with high viral loads), surveillance sensitivity was achieved using 260 fish in pools of 10 for a total of 26 tests, or 200 fish in pools of 5 for 40 tests. Surveillance sensitivity could be maximized even when there was a reduction in pooled diagnostic sensitivity compared to diagnostic sensitivity for individual fish by increasing the sample size. Pooled sensitivity was influenced by the prevalence and variable virus load among fish with sub-clinical infections. Pooled testing is highly effective when the prevalence is >10% which should be informed by prior knowledge or pooling can be used for a screening test to rapidly identify populations with high prevalence.

Running title

Pooled sensitivity testing for freedom from ISKNV

Keywords

Megalocytivirus, Red sea bream iridovirus (RSIV), *Infectious spleen and kidney necrosis virus* (ISKNV), turbot reddish body virus (TRBIV), pooling, sensitivity (PSe), freedom from infection, surveillance sensitivity

1 INTRODUCTION

The global trade in ornamental fish involves the farming or wild-harvest of freshwater and marine species that are predominantly native to tropical regions. The best estimates indicate that annually 1.5 billion fish are traded throughout the world, comprising about 5000 fish species (Whittington and Chong, 2007; Hensen et al., 2010; Ploeg et al., 2012). Nearly all freshwater ornamental fish are bred in captivity. This is in contrast to marine ornamental fish species, which are captured directly from their native habitats often with unsuitable methods, resulting in the destruction of coral reefs, particularly in Southeast Asia (Monticini 2010). Annually, Australia imports approximately 18 million ornamental fish, predominantly from Indonesia, Singapore and Thailand (O'Sullivan et al., 2008). Since 1985, the ornamental fish industry has experienced an average annual growth rate of 14% and with advances in fish husbandry and breeding techniques, it is expected that these industry figures will grow (Whittington and Chong, 2007; Livengood and Chapman, 2017).

The ornamental fish industry presents a high risk 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). Australia is considered to have a highly regulated biosecurity system with stringent controls, compared with 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.

Belonging to the genus *Megalocytivirus*, ISKNV and a closely related but distinct virus, Red sea bream iridovirus (RSIV) are notifiable to the World Organization for Animal Health (OIE, 2018) and are subject to regulation and notification in many countries (Hick et al., 2016). The third genotype of megalocytivirus (MCV), turbot reddish body virus (TRBIV) is not mentioned by the OIE. Since the initial identification in 1990, MCVs are known to infect over 50 marine and freshwater species of fish and have caused epidemics in farmed food fish, ornamental fish and wild fish populations (Subramanian et al., 2012; Waltzek et al., 2012; Hick et al., 2016). Disease caused by MCVs have occurred most frequently in east and south-east Asia (Subramaniam et al., 2012) and mortality is usually between 20 and 60% (Kurita and Nakajima, 2012). Recently, retrospective studies using novel molecular tools have traced the early emergence of MCVs in ornamental fish from the late 1980s and early 1990s in Australia, USA and Canada (Go et al., 2016; Koda et al., 2018). At present the OIE Manual for Diagnostic Tests for Aquatic Animals does not identify a test suitable for targeted surveillance for MCVs (OIE 2018, version adopted 2012). Of the recommended tests for confirmatory diagnosis, the conventional PCR detects the two listed genotypes, others require specialized reagents (monoclonal antibodies) or not applicable to all ISKNV-like isolates (virus isolation). Real-time PCR (qPCR) assays provide the high sensitivity required for surveillance and are compatible with rapid, high-throughput laboratory methods although there is insufficient validation data to estimate the diagnostic sensitivity and diagnostic specificity of these tests (Gias et al., 2011; Rimmer et al., 2012b; Lin et al., 2017). These assays were considered the best available surveillance tools and were required as part of an emergency disease response situation in Australia (Rimmer et al., 2015; Mohr et al. 2015).

ISKNV is currently considered an exotic pathogen to Australia following intensive biosecurity containment measures undertaken in response to (i) an epidemic mortality event at a land-

based recirculating aquaculture facility producing food fish (Lancaster et al., 2003), and (ii) numerous subclinical detections of the virus at domestic retailers and one ornamental fish breeding facility (Rimmer et al., 2015). In response to these virus detections, the Australian Department of Agriculture and Water Resources (DAWR) published a risk assessment stating the import controls for the importation of freshwater fish belonging to the cichlid, gourami and poeciliid families did not meet Australia's appropriate level of protection for ISKNV (Australian Department of Agriculture 2014b). Since 2016, health certification that demonstrates freedom from infection with ISKNV has been required to import fish belonging to the above mentioned families (Australian Department of Agriculture, 2014a).

Surveillance testing for freedom from infection commonly uses pooling of samples without knowledge of the effect of pooling on the diagnostic characteristics of the test. Pooled sample testing is often based on claims for the high analytical sensitivity of qPCR assays to justify increasing the sample size, hence reducing the cost of testing, which might in turn increase the efficiency of surveillance. However, the pooled sample strategies for aquatic pathogens are seldom evaluated using a suitable pathway to estimate the diagnostic pooled sensitivity and specificity. For the detection of RSIV and ISKNV, there was a general recommendation that tissue samples (spleen and kidney) can be pooled from no more than five juvenile fish (less than 3 cm) (OIE, 2018). The objective of this study was to evaluate the effect of tissue pooling strategies for qPCR assays on testing for freedom from infection with ISKNV at a 2% minimum expected prevalence with 95% confidence.

2 MATERIALS AND METHODS

2.1 Source of ornamental fish

Dwarf gourami (*Trichogaster lalius*) and kissing gourami (*Helostoma temminckii*) were imported to Australia in January and May 2015, prior to the change in Australia's current requirements for demonstrating freedom from infection with ISKNV (Table 1). At the exporting facility overseas, 40 to 75 individual fish of the same species were placed in a plastic bag with

approximately 3 to 5 L of freshwater, filled with oxygen and sealed with rubber bands. Bags of fish were placed in Styrofoam boxes and sealed for air transportation. The transportation time from the exporter to arrival in Australia was approximately 24 hours. Upon arrival at Sydney, Australia, fish were subject to the Australian import conditions. All fish were considered apparently healthy as they passed the pre-quarantine inspection which included health certification from exporting country and a visual inspection on arrival by quarantine services. Normally for the pet fish industry, these fish species would be subject to a 14 day quarantine holding period at an Approved Arrangement site (AA Site) prior to being allowed to be distributed in Australia. For our study, on the day of arrival, fish were transported by road to a Physical Containment 2 certified laboratory that was an AA Site at the University of Sydney, Camden. All fish were euthanized with an overdose of benzocaine ($>120 \text{ mg L}^{-1}$) within 6 hours of arriving at the laboratory under the animal ethics approval from the University of Sydney (approval number 720). They were stored individually at -80°C until the time of sample collection.

2.2 Tissue sample preparation and nucleic acid extraction

Briefly, a tissue sample consisted of up to 0.1 g of combined weight of kidney, liver and spleen that was dissected from one freshly thawed fish. Tissue homogenates were prepared at 1/10 w/v in phosphate buffered saline by bead-beating using the FastPrep System (MP Biomedicals) at 6.0 m s^{-2} for 40 seconds (Rimmer et al., 2012b). For clarification, the homogenate was centrifuged at $855 \times g$ for 10 minutes at room temperature and then the supernatant was stored at -80°C . Samples were thawed at 4°C overnight, vortexed for 10 seconds, then centrifuged ($855 \times g$ for 10 minutes at room temperature). For each sample of tissue homogenate, nucleic acids were purified from a $50 \mu\text{L}$ aliquot of the clarified tissue homogenate using the MagMax-96 Viral Isolation Kit (ThermoFisher Scientific) according to the directions of the manufacturer with the AM1836 deep well program on a MagMax Express-96 magnetic particle processor (ThermoFisher). Extracted nucleic acids were eluted in $75 \mu\text{L}$ of elution buffer and stored at -80°C .

158

159 **2.3 Experiment to determine the analytical sensitivity**

160 The limit of detection of the qPCR assay with the nucleic acid extraction process included was
161 evaluated using ten-fold dilution series of the plasmid pDGIV-MCP1 (Rimmer et al., 2012a)
162 added directly to the qPCR reaction mix, or added to molecular grade water or fish tissue
163 homogenates prior to nucleic acid purification. The fish tissue homogenates were obtained
164 from dwarf gourami (Population 3) that were imported from Singapore and were negative for
165 MCV (Table 1). Previously, the analytical sensitivity of the assay using the plasmid spiked into
166 molecular grade water was 1×10^2 copies (Rimmer et al., 2012a). Based on this, we
167 conservatively began the testing in molecular grade water at 1×10^4 . Once the analytical
168 sensitivity was determined to be 0%, no lower concentrations were tested.

169

170 **2.4 Experiment to determine diagnostic sensitivity of pooled tissue homogenates**

171 Two source populations were used with a different prevalence and quantity of viral DNA from
172 naturally acquired MCV infection as determined by individual tests for MCV (Table 1).
173 Population 1 were kissing gourami with moderate prevalence and moderate viral load and
174 Population 2 were dwarf gourami with 100 % prevalence and high viral load. To confirm the
175 ISKNV genotype, two fish from each population with the highest individual virus load were
176 subjected to conventional PCR assay to amplify other key regions of the MCV genome (e.g.
177 segments of the IRB6 and ATPase genes) that are used for genotyping this genus (OIE, 2018).
178 Sanger sequencing was performed at Australian Genome Research Facility. All fish positive by
179 qPCR assay (targeting the MCP gene segment, Rimmer et al., 2012a) were considered to be
180 infected with ISKNV as the number of bases different to the ISKNV reference sequence
181 (GenBank accession AF371960) was 1 or less.

182 The pooling of clarified homogenates from individual fish was completed separately for each
183 species. The positive and negative tissues for dwarf gourami were sourced from Population #2
184 and #3, respectively (Table 1). The positive and negative tissues for Kissing gourami were

sourced only from Population #1. A list of the positive or negative fish was generated and used to allocate individual tissue samples to pools using the random sequence generator (www.random.org) for preparation of 630 unique pools of 5 or 10 tissue homogenates. The ratio of positive and negative samples in each pool is indicated in Table 2. The rationale for the pattern of positive fish was based on probability theory for prevalence in the range 2% to 20% (Table 2). For example, at 2% prevalence the probability of having more than 3 positive samples in a 5-fish pool or more than 5 positives in a 10-fish pool is negligible. A total of 462 pools were prepared from Population 2 (dwarf gourami) and 168 pools prepared from Population 1 (kissing gourami). We chose an unbalanced design in favour of increasing replication for the treatment corresponding to the most probable condition under which more than one fish contributed to the pool. The implication of the lack of balance is that at least one treatment would have more replicates than other treatments, a condition which was handled in the analysis. Pools were prepared in 1.5 ml tubes using 15 μ l or 20 μ l of each clarified homogenate for pools of 10 or 5, respectively. The individual tissue homogenate was vortexed (10 seconds) and centrifuged at 855 x g for 10 minutes at room temperature before subsampling. Following, the pooled homogenate was vortexed for 10 seconds. Nucleic acid purification and the qPCR test for MCPs was conducted as described in Section 2.2 and 2.5, respectively.

2.5 Quantitative PCR (qPCR)

A qPCR assay targeting a conserved region of the major capsid protein (MCP) gene was used according to the method described by Rimmer et al. (2012a). Briefly, duplicate 25 μ L reactions were prepared using 12.5 μ L Quantitect SYBR Green Master Mix (Qiagen), 7.25 μ L molecular grade water, primers C1073 and C1074 at 500 nM each, and 5 μ L of undiluted nucleic acid template. Quantitative PCR was performed using an Mx3000P Multiplex Quantitative PCR System (Stratagene) with the following conditions: 95°C for 15 min; by 40 cycles of 95°C x 30 s, 62°C x 30 s, 72°C x 30 s, followed by a dissociation curve. Each plate contained positive and negative controls for the tissue processing procedure (known positive and negative tissue homogenates that were handled in the same way as each batch of experimental samples),

extraction controls (known positive and negative tissue homogenates that were included at the stage of nucleic acid purification) and PCR controls (known positive and negative nucleic acids that were included at the time of reaction set up). Standard curves for quantification were generated using the plasmid pDGIV-MCP1 (containing a 694 bp ISKNV MCP sequence specific insert) in molecular grade water at concentrations of 1×10^2 to 1×10^6 copies per reaction (Rimmer et al., 2012a). A fluorescence threshold was calculated using the amplification based threshold algorithm (MxPro, Stratagene) for amplification of the plasmid standard and applied to the experimental samples. A Ct value was assigned to samples as the fractional cycle number where the baseline corrected, ROX normalized SYBR signal first exceeded the threshold. A positive result was assigned if at least 1 replicate had a Ct value associated with an exponential increase in fluorescence and the same melting temperature ($85.0 \pm 0.5^\circ\text{C}$) as the positive control tissue homogenate containing DGIV_2010 (Rimmer et al., 2012a). The number of MCV genome copies per gram of tissue was calculated for positive samples if the quantity could be the interpolated from within the range of the standard curve (plot of the Ct value against the template quantity) where the efficiency was 90-110% (Rimmer et al, 2012a).

2.6 Statistical analysis

The outcome was a positive or negative qPCR assay result for MCV for each of the 630 pooled tissue homogenates, which were treated as experimental units. The data were then analyzed using a generalized mixed-effects model (R Core Team, 2018). Briefly, a unique model was fit for each species, with the following characteristics: the PCR plate was used as a random effect and a three-knot penalized spline of the proportion of positive samples for each of the two pool sizes as a fixed effect. The response variable was assumed to be binomial with a probit link function, chosen based on a comparison of competing link functions using Akaike's Information Criterion. This model form was selected after an extensive exploratory analysis phase. The model was:

$$y_{ijkm} \sim Bi(n_i, p_{ijm}),$$

$$p_{ijm} = \phi^{-1}(\beta_0 + f_i(r_j) + s_i + p_m),$$

where y_{ijk} is the outcome for a single test for replicate k within pool size i and positive rate index j , ϕ^{-1} is the inverse cumulative distribution function of the standard normal distribution, β_0 is a fixed intercept, r_j is the positive rate, f_i is a penalized cubic spline function for each pool size, s_i is the pool size, and $p_m \sim N(0, \sigma^2)$ is a PCR plate-specific random effect.

Next, the fitted model was used to estimate the probability of successful detection as a function of pool size and the number of positive samples contributing to the pool. These predictions were used as the basis of a simulation model to determine the confidence for testing for freedom from ISKNV in a population of fish at a minimum expected prevalence of 2%. Briefly, we simulated the process of selecting n units from a population, and then: (i) simulated testing the units individually, assuming 100% sensitivity and 100% specificity, to serve as a benchmark; (ii) simulated pooling the units into 30 pools of size 5, then predicting the test outcome for each pool as a function of predicted ratio of positive samples using the model, and (iii) simulated pooling the units into 15 pools of size 10, then predicting the test outcome for each pool as a function of its contamination rate using the model. This process was repeated 10 000 times for n ranging from 150 to 300. The outcome of the exercise was the estimated surveillance sensitivity of the test as a function of the sample size n and the pool size (1, 5, or 10).

In other words, the goal of the study was to assess the diagnostic sensitivity of the test when applied to pools of samples generated from populations with different prevalence of infection. We approached this efficiently by separating the challenges of (i) preparing pools based on the results of tests on individual fish and (ii) performing an experiment that let us model the diagnostic sensitivity for a given pool of fish as a function of the number of infected fish contributing to the pool. We then conducted a simulation experiment in which we simulated taking pooled samples from a population with known prevalence and predicting the pool-level sensitivity from the model. We aggregated these sensitivities across the simulated sample to obtain the total diagnostic sensitivity. We tried different numbers of pooled samples in order to see how many were required to achieve the nominal sensitivity.

3 RESULTS

3.1 Limit of detection

The analytical sensitivity for the detection of ISKNV assessed using a plasmid DNA standard was reduced by two orders of magnitude when the nucleic acid purification procedure was considered as part of the testing procedure (Table 3). Compared to the limit of detection between 10 and 100 copies of template per reaction when used directly in qPCR, addition of the plasmid standard to molecular grade water and fish tissue homogenates prior the nucleic acid purification procedure used in this study reduced the limit of detection in the order of 100-fold and 1000-fold, respectively (Table 3).

3.2 Sensitivity of qPCR for pooled tissue homogenates

For Population 1, the prevalence of ISKNV was 66% and a moderate virus load ranging between with a median of 6.2×10^4 copies (Table 1, Figure 1). Comparatively, the prevalence of infection for Population 2 was 100% and a median load of 9.6×10^6 copies (Table 1, Figure 1). This provided authentic pooled samples that reflected the sample matrix and virus load of apparently healthy imported ornamental fish.

The sensitivity of the qPCR assay to detect pools including one or more positive fish (pooled sensitivity) was substantially reduced compared to testing individually (Figure 2 and 3). This reflected the ratio of positive to negative samples contributing to the pool. The generalized mixed-effects model identified a significant impact of the population from which the samples were sourced on the pooled sensitivity ($p < 0.01$). Higher pooled sample sensitivity was obtained when pools were prepared using Population 2 (100% prevalence and relatively higher viral loads) compared to Population 1 (66% prevalence and moderate viral loads) (Figure 2). The raw (unmodelled) sensitivity for a single positive sample in a pool of 5 was 37.5% (95% CI, 17.4 - 61.7%) and 75% (95% CI, 60.9 - 86.9%) for Population 1 and Population 2, respectively (Figure 2). For a single positive in a pool of 10 the raw (unmodelled) sensitivity was 31.3% (95% CI, 13.1 - 55.6%) and 72.7% (95% CI, 58.4 - 84.1%) for Population 1 and Population 2, respectively

(Figure 2). Therefore, predictions for pooled sensitivity derived from the laboratory testing were stratified for the two populations.

The different pooled sensitivity for the two populations was extremely influential in determining the surveillance sensitivity (Table 4, Figure 3). Simulations for tests of a population for freedom from infection were undertaken according to OIE guidelines for 95% confidence of detecting a minimum expected prevalence of 2% with the assumption that the qPCR tests of individual fish provided 100% sensitivity and 100% specificity. For Population 1, the nominal surveillance sensitivity could be achieved using 40 pools of 5 fish (200 fish, 40 tests) or 26 pools of size 10 (260 fish, 26 tests) in place of 150 tests of individual fish (Table 4, Figure 3). However, the surveillance sensitivity for Population 2 (with 66% prevalence and moderate viral loads) did not reach the desired level of confidence for any sample size with pools of 5 or 10 fish (Table 4, Figure 3).

4 DISCUSSION

This study highlighted the important influence of target population characteristics including prevalence of infection and pathogen load which result in variable sensitivity for pooled testing strategies. Infection with MCV has a complex pathogenesis whereby host and environmental factors overlay at all stages of infection to produce wide ranging variation in the prevalence, virus distribution and load such that pooled sensitivity cannot be provided as a single estimate that is applicable in all scenarios. The two source populations used to provide naturally infected tissues for this study were each easily identified as a biosecurity risk using individual fish testing, yet had contrasting outcomes when used to generate samples to estimate pooled sensitivity. For Population 1 (kissing gourami) the source population had 66% prevalence and a median copy number of 6.2×10^4 MCV DNA copies per mg, which was close to the limit of detection of the assay, when nucleic acid purification was considered. Population 2 (dwarf gourami) had 100% infection and a substantially higher viral load (median copy number of 9.6×10^6 ISKNV DNA copies per mg), reflecting the variation seen in MCV load at different times post-infection and other risk factors such as the age and general health of the host (Sano et al.,

2002). In scenarios where these viral loads were presented as pools that would arise under a hypothetical prevalence of 2% (and the desired surveillance sensitivity was 95%), it was not possible to achieve the desired surveillance sensitivity using pools of 5 or more for Population 1. For the fish from Population 2, the surveillance sensitivity was achieved using 260 fish in pools of 10 for a total of 26 tests, or 200 fish in pools of 5 for a total of 40 tests. Pooled testing was predicted to be highly effective for a prevalence >10% with MCV loads presented in each source population. Pooled testing could be cost effective when informed by prior knowledge about expected prevalence as in risk based surveillance or be used as a screening test to rapidly identify populations with high prevalence of infection.

This study showed that there is a need to consider different risk factors, such as fish species and stages of infection as separate scenarios for surveillance testing. The OIE Aquatic Manual for RSIV (2018 adopted in 2012) states that spleen and kidney can be pooled from no more than five juvenile fish (less than 3 cm). This study showed that a general recommendation for pooling is not able to provide a predictable surveillance sensitivity. Pooling lowered the surveillance sensitivity below that predicted from validation data derived from individual samples. We demonstrated analytical sensitivity of qPCR was much lower when the inefficiency of nucleic acid purification was considered with the plasmid contained in a biological matrix (e.g. fish tissues) instead of molecular grade water. This indicated that assessment of the analytical characteristics of qPCR surveillance systems should account for the entire laboratory procedure (e.g. tissue preparation, nucleic acid extraction and PCR performance). Pooled samples were impacted by the amount of virus DNA in target tissues of the infected individuals. In practice, this can be close to the limit of detection at different stages of infection in acutely and subclinically infected fish. Fish species-specific impacts on sensitivity could also require further evaluation to impact test performance. From this study, pooling should not be considered in target populations when disease is expected to be rare as the surveillance sensitivity can drop to below expected standards. Prior to Australia requiring batch testing for ISKNV, ornamental gourami under quarantine had an average 24.1% ($\pm$ 6% SEM) of fish infected with the virus (Rimmer et al., 2015). The minimum and maximum copy number for these positive fish populations were reported as $<2.7 \times 10^2$ and 5.89×10^8 per mg of fish tissue,

351 respectively (Rimmer et al., 2015). The results from this study would indicate that pooled
352 sample testing for freedom from infection would not be appropriate at the OIE standard of less
353 than 2% prevalence.

354 The imperative to use pooling is highest in the surveillance scenario compared to
355 confirmatory diagnosis as the sample size and cost of the procedure is large. The lower pooled
356 sensitivity compared to diagnostic sensitivity for individual fish could be overcome when the
357 sample size can be feasibly increased above 150. This can reduce laboratory costs with a lower
358 number of PCR tests. For the current study, the fewest tests was 26 requiring the sacrifice and
359 costs of processing 260 fish. Virus loads encountered in the different scenarios are considerably
360 different and evaluation of pooled sensitivity on diagnostic clinical samples would provide
361 misleading results if interpreted for surveillance. In other words, there is a very high probability
362 of detecting diseased fish used pooled testing during an outbreak. This was demonstrated in
363 Atlantic salmon (*Salmo salar*) collected from a farm that was experiencing an outbreak with the
364 OIE-listed pathogen, infectious salmon anaemia virus (ISAV). Hall et al. (2013) reported a
365 nominal drop in pooled sensitivity (at 2% true prevalence) from 90% (95% CI: 76-98) to 83%
366 (95% CI: 70-91%) when testing for ISAV in pools of five using quantitative reverse transcriptase
367 PCR (qRT-PCR). However, the differences in the pooled sensitivity compared to individual
368 testing disappeared as prevalence of infection increased (Hall et al., 2013). Thus, the probability
369 of detecting ISAV in disease outbreak scenarios using pooled tests was reported as very high
370 (>95%) (Hall et al., 2013). Similar results were obtained for the detection of *Salmonid alphavirus*
371 (also OIE-listed), which causes pancreas disease in Atlantic salmon. Under a theoretical testing
372 scenario for freedom from infection testing (<2% true prevalence), Hall et al. (2014) reported a
373 considerable drop in pooled sensitivity to 6% (95% CI: 3-18%) with pools of five tested using
374 qRT-PCR. However, if the true prevalence was 80% (e.g. during an outbreak), the sensitivity of
375 the pooled test was 54% (95% CI: 48-62%) (Hall et al., 2014). In agreement with Hall et al.
376 (2014), our study showed that individual testing for freedom from infection was the only
377 appropriate approach for detecting 2% prevalence in a population of apparently healthy fish.

In agreement with our study, 26 pools of ten were needed to meet the freedom from infection for detecting the parasite *Bonamia ostreae* in New Zealand flat oysters (*Ostrea chilensis*) (Lane et al., 2017). Using the 18S rRNA gene assay, other combinations that met the OIE guidelines for freedom from infection included 50 pools of three, and 30 pools of five (Lane et al., 2017). *B. ostreae* is an emerging aquatic pathogen recently detected in New Zealand in 2015. It has caused significant disease events in wild and farmed flat oysters in Europe and North America (Elston et al., 1986). Lane et al., (2017) compared two qPCR assay with SYBR Green detection chemistry with a focus on maximizing sensitivity for early detection surveillance when there were no clinical signs or lesions to support detection.

In addition to the high cost of the tests, the imperative to pool samples for PCR is driven by the large sample size required when aiming to demonstrate freedom from infection. East (2008) identified that pooling strategies were desirable when the value of individuals was low, the prevalence was high and at stages in the pathogenesis of the infection when there was high pathogen load. However, it was concluded based on extrapolation of analytical data that pooling would not have been appropriate in a national survey to evaluate freedom from infection in response to a white spot syndrome virus (WSSV) incursion in Australia in 2000 (East et al., 2004). The evaluation of the impact of pooling on test reliability was prompted by the need to reduce the cost of qPCR tests on large sample sizes needed for a sensitive survey (East et al., 2004).

We need to improve our understanding of the epidemiology of MCV infections with particular reference to host and environmental factors that affect the transmission of the virus and the differentiation of subclinical infection and disease. The first description of a MCV (RSIV genotype) was in 1990 in Japan from a marine species, red seabream (*Pagrus major*) (Inouye et al., 1992). Previously, ISKNV was considered a freshwater pathogen mostly of ornamental fish and the important aquaculture species mandarin fish (*Siniperca chuatsi*) whilst RSIV mostly infected marine food fish (Kurita and Nakajima, 2012). Current evidence indicates that we must consider the three genotypes of ISKNV (i.e. RSIV, ISKNV and TRBIV) as potentially globally distributed pathogens, each able to infect fish in a variety of environments (Fusianto et al.,

2019). For example, ISKNV has been reported as the cause of significant mortality at marine farms holding barramundi (*Lates calcarifer*) (Dong et al., 2017) whilst the geographic range is expanded with detections in ornamental fish in Brazil (de Lucca et al., 2018), Germany (Jung-Schroers et al. 2016) and the USA (Subramaniam et al. 2016). TRBIV was recently reported in a marine food fish (starry flounder, *Platichthys stellatus*) in Korea (Jin et al., 2018; Woong et al., 2018) and from archived clinical cases of freshwater ornamental fish from the late 1980s and early 1990s in the USA (Koda et al., 2018), Canada and Australia (Go et al., 2016). Further, Liu et al (2018) reported that the original freshwater host of ISKNV, mandarin fish is now frequently affected by RSIV in China. The sequence variation between the three genotypes of MCV requires PCR assay validation for each genotype, whilst at present there is insufficient validation data to predict the diagnostic sensitivity of MCV assays for any one genotype (OIE, 2018). With an understanding that subclinical MCV infection can exist at low prevalence and low viral load, where the risk of transmission provides a biosecurity risk pathway, pooled sample testing will not provide the required surveillance sensitivity.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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Table 1. Summary of imported ornamental fish tested individually by qPCR for the detection of MCV and confirmed to be infected with *Infectious spleen and kidney necrosis virus*.

Population No.	Common name	Homogenate usage	Export country	Date received	Weight ¹ (g)	Total length ¹ (mm)	Proportion of positive fish	Percent positive (95% CI) ²
1	Kissing gourami	Positive and negative tissues for pooling experiment	Singapore	May 2015	2.2 ± 0.64	50.2 ± 5.12	114/172	66.3 (58.7 - 73.3)
2	Dwarf gourami	Positive tissues for pooling experiment	Thailand	May 2015	1.29 ± 0.33	39.12 ± 3.32	178/178	100 (97.9-100)
3	Dwarf gourami	Negative tissues for analytical sensitivity and pooling experiments	Singapore	January 2015	3.0 ± 0.5	49.2 ± 3.1	0/184	0 (0 - 2)

¹ mean ± standard deviation

²95% binomial confidence intervals (CI) were calculated

588 Table 2: Sample sizes for the pooling of tissue homogenates from ornamental fish for
 589 the detection of *Infectious spleen and kidney necrosis virus*.

Size	Pool No. of positive homogenates	Expected frequency ¹			No. of pools prepared	
		Prevalence			Population 1	Population 2
		2%	10%	20%	(Kissing gourami)	(Dwarf gourami) ²
5	0	0.90	0.59	0.32	16	44
	1	0.10	0.34	0.42	16	44
	2	0	0.07	0.21	20	55
	3	0	0.01	0.05	16	44
10	0	0.81	0.34	0.10	16	44
	1	0.18	0.40	0.27	16	44
	2	0.01	0.20	0.31	20	55
	3	0	0.05	0.21	16	44
	4	0	0.01	0.09	16	44
	5	0	0	0.02	16	44

590 ¹ The expected frequency column provided the probability that a randomly-selected pool of
 591 given size (5 or 10; major row) taken from a population with the given prevalence (column)
 592 would yield the given number of positive homogenates (0 – 3 or 0 – 5; minor row).

593 ² Negative tissue homogenates were sourced from Population 3 (Table 1)

594

Table 3. Analytical sensitivity of the qPCR assay for detection of *Megalocytivirus* using pDGIV-MCP1 DNA plasmid added directly to the qPCR reaction mix or prior to nucleic acid purification into molecular grade water or fish tissue homogenate (n represents the number of tests).

Plasmid copies/ qPCR	Plasmid added to	Plasmid added to sample matrix prior to nucleic acid purification ^{1,2}			
	qPCR reaction mix (n = 18) ¹	Molecular grade water (n=9)		Tissue homogenate (n=18)	
	Percent positive detection (95% CI)	Mean copy number detected	Percent positive detection (95% CI)	Mean copy number detected	Percent positive detection (95% CI)
1x10 ⁶	100 (81.5-100)	not done	not done	1.04x10 ³	100 (81.5-100)
1x10 ⁵	100 (81.5-100)	not done	not done	1.63x10 ²	88.9 (65.3-98.6)
1x10 ⁴	100 (81.5-100)	7.98x10 ¹	100 (66.4-100)	2.33x10 ¹	77.8 (52.4-93.6)
1x10 ³	100 (81.5-100)	6.13x10 ⁰	77.8 (40.0-97.2)	1.45x10 ⁰	27.8 (9.7-53.5)
1x10 ²	100 (81.5-100)	0	0 (0-33.6)	0	0 (0-18.5)
1x10 ¹	94.4 (74.2-99.0)	not done	not done	not done	not done

¹ 95% binomial confidence intervals (CI) were calculated

² Plasmid DNA was spiked into 50 µL samples prior to nucleic acid extraction at a concentration that would provide the copies if there was 100% extraction efficiency.

Table 4. Surveillance sensitivity using different combinations of sample size and pool size from a population with a prevalence of 2% for the detection of *Infectious spleen and kidney necrosis virus*. The shaded boxes indicate a pool and sample size combination that achieved sensitivity greater than 95%.

Sample Size	Pool size					
	Population 1			Population 2		
	Kissing gourami			Dwarf gourami		
	1	5	10	1	5	10
150	0.96	0.48	0.50	0.96	0.89	0.80
160	0.97	0.50	0.52	0.97	0.91	0.83
170	0.98	0.52	0.54	0.98	0.92	0.85
180	0.98	0.54	0.56	0.98	0.93	0.86
190	0.99	0.56	0.58	0.99	0.94	0.88
200	0.99	0.58	0.60	0.99	0.95	0.89
210	0.99	0.60	0.62	0.99	0.96	0.90
220	0.99	0.62	0.63	0.99	0.97	0.92
230	1.00	0.64	0.65	0.99	0.97	0.93
240	1.00	0.65	0.67	1.00	0.98	0.93
250	1.00	0.67	0.68	1.00	0.98	0.94
260	1.00	0.68	0.70	1.00	0.98	0.95
270	1.00	0.69	0.71	1.00	0.99	0.95
280	1.00	0.71	0.72	1.00	0.99	0.96
290	1.00	0.72	0.74	1.00	0.99	0.97

300	1.00	0.73	0.75	1.00	0.99	0.97

Figure 1. Quantitative PCR results for the individual testing of ornamental fish from Population 1 (kissing gourami) and Population 2 (dwarf gourami) for the positive detections of *Infectious spleen and kidney necrosis virus*.

Figure 2. The generalized mixed-effects model for the diagnostic sensitivity experiment for the detection of *Megalocytivirus*. Tissues homogenates from Population 1 (kissing gourami) and Population 2 (dwarf gourami) were pooled as shown in Table 2.

Figure 3. Simulation using the pooled sample sensitivity for Population 1 (kissing gourami) and Population 2 (dwarf gourami) predicted by the generalized mixed-effects model to determine the probability of detecting an infection at 2% prevalence using different combinations of pool size and sample size.

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