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Design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Acknowledgments

The significant contributions by the workshop participants through preparation and presentation of comprehensive information concerning experimental design are greatly appreciated as are the open and frank discussions that were held during the workshops. Without this level of collaboration the workshop outputs and value would have been severely limited.

Abbreviations

AAHL	Australian Animal Health Laboratory
AbHV	Abalone herpesvirus
CERC	Canada Excellence Research Chair
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	Cycle threshold
DAS	Diagnostic accuracy studies
DAWR	Australian Federal Government Department of Agriculture and Water Resources
DSe	Diagnostic sensitivity
DSp	Diagnostic specificity
DTE	Diagnostic test evaluation
EHNV	Epizootic haematopoietic necrosis virus
ELISA	Enzyme-linked immunosorbent assay
FRDC	Fisheries Research and Development Corporation
IFAT	Immunofluorescent antibody test
i.m.	intramuscular
ISKNV	Infectious spleen and kidney necrosis virus
LAF	AAHL Large Animal Facility
MedCalc	A statistical software package designed for the biomedical sciences
OIE	Office International des Epizooties - the World Organisation for Animal Health
OsHV	Ostreid herpesvirus
OsHV-1	Ostreid herpesvirus Type 1
PSe	Diagnostic sensitivity of a test on pools
PSp	Diagnostic specificity of a test on pools
QUADAS	Quality assessment tool for diagnostic accuracy studies
RNA	Ribonucleic acid
RSIV	Red sea bream iridovirus
SKF-9	Cell line developed from larval knifejaw (*Oplegnathus punctatus*)
SCAAH	Sub-committee of Aquatic Animal Health – a sub-committee of the Australian Federal Government's Animal Health Committee
WSSV	White spot syndrome virus
YHV	Yellow head virus

Executive Summary

What the report is about

It is well-recognised that validation of diagnostic tests for diseases that are exotic to Australia is hampered by the lack of reference populations that are infected with the specific disease agent. Experimental infectivity studies provide one approach to address this gap. However, there is a need for design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. One way forward is to establish a writing group to draft the design standards with the aim to publish in a peer-reviewed journal to obtain a wide distribution to aquatic animal health specialists.

Background

In consultation with Prof Ian Gardner (OIE working groups on principles and approaches to validation of diagnostic tests), Dr Ingo Ernst (Director, Aquatic pest and health policy, Department of Agriculture and Water Resources; President, OIE Aquatic Animal Disease Commission), Prof Richard Whittington, Dr Paul Hick (University of Sydney, OIE Reference Laboratory for EHNV/*Ranavirus*), Dr Nick Moody and Dr Mark Crane (CSIRO AAHL Fish Diseases Laboratory, OIE Reference Laboratory for EHNV/*Ranavirus*, YHV and AbHV) discussed the need for design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. Being a priority for DAWR, funding was obtained, via FRDC, for a project to address this need.

Aims/objectives

1. Evaluate (a) design criteria reported for diagnostic studies of OIE-listed diseases of finfish, molluscs and crustaceans and (b) test design and accuracy recommendations from OIE, peer-reviewed literature in aquatic animal health and other sources (e.g. aquatic animal health experts).
2. Develop a list of gaps or inconsistencies in current design criteria and reporting for OIE-listed diseases in aquatic species, based on collected recommendations from Objective 1.
3. Compile consensus-based standards in an applicable format for diagnostic studies in finfish, molluscs and crustaceans for use by research journals and laboratories.

Subsequently, a fourth objective was included as part of the project:

4. Develop experimental protocols for the determination of pool-level diagnostic sensitivity for the qPCR detection of sub-clinical infections involving specific pathogens (WSSV, YHV, *Megalocytivirus*), with input from an expert aquatic epidemiologist.

Methodology

A systematic literature review of studies designed to address test sensitivity and specificity for the OIE-listed aquatic animal diseases was undertaken. In a workshop format, invited experts exchanged and discussed the information on the use of experimental and field studies to examine the performance of diagnostic tests for aquatic animal diseases, particularly OIE-listed diseases. The workshop participants discussed gaps or inconsistencies between recommendations and current reporting, and mitigation strategies to identify guidelines applicable to aquaculture research. All compiled recommendations adapted to aquatic animal research were formatted to a practical and easy-to-apply checklist tool for publication.

In addition, the fourth objective, concerning development of experimental protocols to determine pool-level sensitivity of diagnostic tests for detecting sub-clinical infections, was addressed during a third

workshop during which experts in aquatic epidemiology and test validation discussed appropriate protocols that would lead to the production of robust data that could be used to determine pool-level sensitivity for three specified agents, White spot syndrome virus (WSSV), Yellow head virus (YHV) and *Megalocytivirus*.

Results/key findings

The results for objectives 1-3 are encompassed in the publication:
Laurin E, Thakur KK, Gardner IA, Hick P, Moody NJG, Crane MSJ, Ernst I. 2018. Design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. *J Fish Dis* **41:** 729-749.

A third workshop was convened at CSIRO-AAHL, Geelong on 22-26 June 2018 to progress development of experimental protocols for the determination of pool-level diagnostic sensitivity for specific pathogens and tests, with input from an expert aquatic epidemiologist (Objective 4).

The experts convened to discuss the detailed methods to be used to address the objectives of the Commonwealth of Australia (DAWR) Contract 78260: "*To develop and validate fit-for-purpose levels of pooling for surveillance of aquatic animal pathogens of national and trade significance. The key outcome for each disease will be an estimate of pool-level diagnostic sensitivity for the disease and applicable method*".

Implications for relevant stakeholders

Important metrics for consideration at the design phase of diagnostic accuracy studies (DAS), viz. study purpose, targeted disease state, selection of appropriate samples and specimens, laboratory analytical methods, statistical methods and data interpretation, have been identified and published. Following standards for designing DAS will assist in appropriate test selection for specific testing purposes and minimize the risk of reporting biased estimates of diagnostic sensitivity and specificity.

With respect to pool-level sensitivity, using the developed protocols in DAS will ensure that the effect of pooling samples for the specified disease agents and hosts on diagnostic sensitivity will be clearly defined.

Recommendations

The current lack of quality standards makes it difficult to assess the appropriateness of diagnostic tests for a given purpose. Standardisation of the design stage of studies aimed at the development/assessment of diagnostic tests for aquatic animal diseases is recommended, taking into account a range of factors such as the purpose of the test, the target population, disease status, environmental conditions of the study, sample selection, experimental challenge design, analytical methods and interpretation.

Keywords

Aquatic animals, design standards, diagnostic accuracy, sensitivity and specificity

Introduction

It is well-recognised that validation of diagnostic tests for diseases that are exotic to Australia is hampered by the lack of reference populations that are infected with the specific disease agent. Experimental infectivity studies provide one approach to address this gap. In consultation with Prof Ian Gardner (OIE working groups on principles and approaches to validation of diagnostic tests), Dr lngo Ernst (Director, Aquatic pest and health policy, Department of Agriculture and Water Resources; President, OIE Aquatic Animal Disease Commission), Prof Richard Whittington, Dr Paul Hick (University of Sydney, OIE Reference Laboratory for EHNV/*Ranavirus*), Dr Nick Moody and Dr Mark Crane (CSIRO AAHL Fish Diseases Laboratory, OIE Reference Laboratory for EHNV/*Ranavirus*, YHV and AbHV) discussed the need for design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. One way forward was to establish a writing group to draft the design standards for distribution to aquatic animal disease laboratories in Australia (and possibly overseas) for review and comment prior to finalisation and publication.

In aquatic animals, experimental studies are often used to study the pathogenicity of an etiologic agent; to study interactions between the host, pathogen, and environment; and to evaluate the comparative performance of existing and novel diagnostics. In the latter case, specimens are sometimes collected from animals at different stages of infection post-challenge to demonstrate temporal changes in diagnostic sensitivity post-challenge. Diagnostic accuracy studies should be designed to assess a test's fitness for a designated purpose, and facilitate publication in peer-reviewed journals. Reported studies should include clear descriptions of purpose and intended application, and authors should discuss the limitations of their results in the context of other available or currently-used tests to facilitate informed decisions by end-users. In human medical research, standards exist for various aspects of diagnostic test research, including guidelines for accurate and transparent reporting and for quality assessment of methodologies in systematic reviews of diagnostic-accuracy studies. Similar standards are required for animal, including aquatic animal, research. It is anticipated that the established criteria, from human clinical research for evaluating diagnostic accuracy studies, can be adapted to develop a standardized set of criteria for the design of field and experimental studies to assess diagnostic accuracy for infectious diseases in aquatic animals.

To address the extra, fourth objective concerning development of experimental protocols to determine pool-level sensitivity of diagnostic tests for detecting sub-clinical infections, a third workshop was required. During this workshop, experts in aquatic epidemiology and test validation were consulted to ensure developed protocols were sufficient to produce robust data that could be used to determine pool-level sensitivity for three specified agents viz. White spot syndrome virus (WSSV), Yellow head virus (YHV) and *Megalocytivirus*.

Objectives

1. Evaluate (a) design criteria reported for diagnostic studies of OIE-listed diseases of finfish, molluscs and crustaceans and (b) test design and accuracy recommendations from OIE, peer-reviewed literature in aquatic animal health and other sources (e.g. aquatic animal health experts).
2. Develop a list of gaps or inconsistencies in current design criteria and reporting for OIE-listed diseases in aquatic species, based on collected recommendations from Objective 1.
3. Compile consensus-based standards in an applicable format for diagnostic studies in finfish, molluscs and crustaceans for use by research journals and laboratories.

Subsequently, a fourth objective was included as part of the project:

4. Develop experimental protocols for the determination of pool-level diagnostic sensitivity for the qPCR detection of sub-clinical infections involving specific pathogens (WSSV, YHV, *Megalocytivirus*), with input from an expert aquatic epidemiologist.

Method

Objectives 1-3

Preliminary work included a literature search to obtain information on the use of experimental and field studies to examine the performance of diagnostic tests for aquatic animal diseases, particularly OIE-listed diseases. For papers identified, studies were categorized as analytical or diagnostic test-accuracy studies. Diagnostic test-accuracy studies were further categorized by study design and purpose (screening, disease confirmation, targeted surveillance, other, or not specified). Two reviewers (Emilie Laurin, Ian Gardner) were involved in the classification and evaluation of identified papers, to minimize bias. Diagnostic studies were determined from their explicit mention of, or the ability to easily extrapolate, diagnostic accuracy measures (e.g., Diagnostic Sensitivity (DSe) and Diagnostic Specificity (DSp)). These papers were evaluated to record information on any reported design criteria and diagnostic test accuracy methods. Information on currently recommended applicable guidelines from human clinical medicine, from terrestrial and aquatic guidelines described in OIE test-validation manuals, applicable reporting guidelines for animal health research, papers describing design considerations were collected and discussed, in a workshop setting, with a panel of aquatic animal health scientists for expert opinion. The workshop participants discussed gaps or inconsistencies between recommendations and current reporting, and mitigation strategies to identify guidelines applicable to aquaculture research. All compiled recommendations adapted to aquatic animal research were formatted to a practical and easy-to-apply checklist tool for publication.

The following experts were invited to the first writing group workshop held in Canberra, 29-30 March 2016:

Prof Ian Gardner, Canada Excellence Research Chair - Aquatic Epidemiology Atlantic Veterinary College, University of Prince Edward Island, Canada

Dr Emilie Laurin, Atlantic Veterinary College, CERC/Dept of Health Management, University of Prince Edward Island, Canada

Dr Paul Hick, Senior Lecturer in Veterinary Virology (Aquatic, Farm Animal and Ecosystem Health) Farm Animal Health, Faculty of Veterinary Science, The University of Sydney, Camden, NSW.

Prof Richard Whittington, Chair, Farm Animal Health, Faculty of Veterinary Science, The University of Sydney, Camden, NSW.

Dr Charles Caraguel**,** Veterinary Epidemiology & Aquatic Population Health, School of Animal & Veterinary Sciences, The University of Adelaide, Roseworthy Campus, SA.

Dr Ingo Ernst, Director Aquatic Pest and Health Policy, Animal Division, Department of Agriculture and Water Resources, Canberra, ACT.

Dr Nick Moody, Senior Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

Dr Mark Crane, Senior Principal Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

All experts accepted the invitation but prior to the first writing group workshop, Prof Richard Whittington had to withdraw due to other work commitments.

i. Workshop I (Canberra, 2016)

Workshop agenda is provided in Appendix I.

The first objective of this study was to evaluate design information reported for Diagnostic Test Evaluation (DTE) studies of OIE-listed aquatic diseases (10 finfish, 7 molluscan, 9 crustacean, and 2

amphibian). Gaps were identified by evaluating and collating test design and accuracy recommendations, and consensus-based standards were compiled. Five hundred and fifty-six studies underwent initial screening, with 262 progressing to full-paper analysis. Of 56 diagnostic papers identified, 41 had field applications, 8 experimental, and 7 both field and experimental. The structured triad for DTE studies for chronic infectious diseases in terrestrial animals described by Nielsen *et al*. (2011) was modified for aquatic-animal relevance, and a modified list of specific design criteria incorporating the four domains of QUADAS-2 (patient selection, index test(s), reference standard, flow and timing) was compiled and expanded to include experimental challenge models. Criteria for designing DTE studies were proposed using examples from the literature and presented in an applicable flowchart, listing risks-of-failure for each focal point and suggesting mitigation steps, highlighting specific applications for field-based and experimental challenge-based studies.

Tasks were identified as follows:

A. Diagnostic Test Evaluation (DTE) objectives:
    1. Purposes: demonstration of freedom, confirmation of clinical case (note: tests' comparison for given purpose)
    2. Infection dynamics: individual & population level
    3. Target condition: individual & population level
    4. Test Under Evaluation

B. DTE Design
    1. Experimental challenge
        a. Subjects
        b. Inocula
        c. Challenge/Model
        d. Housing/caring
        e. Sampling timeframe, protocols
        f. Subsection to be refined…
    2. Field-based sampling
        a. Subject selection
        b. Tests Under Evaluation
        c. Reference standard or lack thereof
        d. Flow and timing

Pre-appraisal tool

Some time was spent discussing experimental design for investigating the effect of pooling on DSe which was considered important when designing surveillance programs and would be discussed at the follow-up workshop.

During the period between the two workshops a manuscript entitled, "Design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals" was completed and submitted for publication on March 3, 2017.

ii. Workshop II (Geelong, 2017)

The second workshop was convened at CSIRO-AAHL, Geelong on March 16-17, 2017. The focus of this workshop was the effect of pooling on test sensitivity.

Participants:

Prof Ian Gardner, Canada Excellence Research Chair - Aquatic Epidemiology Atlantic Veterinary College, University of Prince Edward Island, Canada

Dr Emilie Laurin, Atlantic Veterinary College, CERC/Dept of Health Management, University of Prince Edward Island, Canada

Dr Paul Hick, Senior Lecturer in Veterinary Virology (Aquatic, Farm Animal and Ecosystem Health) Farm Animal Health, Faculty of Veterinary Science, The University of Sydney, Camden, NSW.

Dr Ingo Ernst, Director Aquatic Pest and Health Policy, Animal Division, Department of Agriculture and Water Resources, Canberra, ACT.

Dr Nick Moody, Senior Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

Dr Mark Crane, Senior Principal Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

Dr Axel Colling, Principal Research Consultant (test validation), CSIRO-AAHL

Ingo was only present for one of the days and so we discussed issues that were considered the highest priority first, particularly those for which we needed explicit guidance from him.

Also, Emilie was unable to come to the workshop in person but was able to participate for about the first 2 hours (9 to 11am Geelong) on both days via Skype.

The workshop agenda is provided in Appendix II.

The second workshop considered the effect of pooling on test sensitivity:

- Guidelines for finfish, amphibians, molluscs, crustaceans; OIE-chapter based, etc.
- Addition of published and unpublished data

Questions arising from literature search:

- Where does the common 5 or 10 samples per pool designation come from? Arbitrary? Statistical? Evidence for certain species?
- Hyatt *et al*. (2007) gives recommendations for pool size based on spiked swab experiment, but does this adequately mimic natural environment and expected outcome in field?
- Papers use different techniques for homogenization of pools? Is there a particular method in general by sample type that is adequate? Or statistical evidence for effect of different homogenization techniques on diagnostic test accuracy outcome? Pooling done before or after storage? And recommended techniques for changing homogenization in each case?
- Minimum required information to report on pooling methods?
- Hall papers on PSe and PSp? (Hall *et al*., 2013; 2014)
- Tissue samples vs swabs

By the end of the meeting it was decided that some extra experimental work was warranted. This will be progressed for the top priority disease agents, viz., white spot syndrome virus of crustaceans (crustacean pathogen) and, if possible, Ostreid herpesvirus-1 µVar (mollusc pathogen) and *Megalocytivirus* (finfish pathogen), where there were samples already available thus eliminating the necessity of collecting further wild-caught or experimentally infected material. This extra work would require project extension. Ingo Ernst agreed that any surplus funds from the current project could be utilised for the extension.

The following abstract was prepared for the 2017 EAFP International Conference:

Emilie Laurin, Paul Hick, Ian A. Gardner, Nicholas J. G. Moody, Mark St. J. Crane, Ingo Ernst, David Cummins. To pool or not to pool? Guidelines for pooling samples for tests with different diagnostic end-purposes in aquatic animals.

iii. Workshop III (Geelong, 2018)

A third workshop was convened at CSIRO-AAHL, Geelong on 22-26 June 2018 to progress development of experimental protocols for the determination of pool-level diagnostic sensitivity for specific pathogens and tests, with input from an expert aquatic epidemiologist.

The following experts were invited to discuss experimental methodology to determine pool-level diagnostic sensitivity leading to development of experimental design:

Prof Ian Gardner, Canada Excellence Research Chair - Aquatic Epidemiology Atlantic Veterinary College, University of Prince Edward Island, Canada.

Dr Yuko Hood, Aquatic Pest and Health Policy, Animal Division, Department of Agriculture and Water Resources, Canberra, ACT (participated on 26 June 2018 only).

Dr Nick Moody, Principal Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

Dr Peter Mohr, Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

Dr Mark Crane, Senior Principal Research Scientist, CSIRO AAHL Fish Diseases Laboratory, Geelong, VIC.

Dr Axel Colling, Principal Research Consultant, CSIRO AAHL Veterinary Investigation and Epidemiology, Geelong, VIC.

The experts convened to discuss the detailed methods to be used to address the objectives of the **Commonwealth of Australia (DAWR) Contract 78260: "*To develop and validate fit-for-purpose levels of pooling for surveillance of aquatic animal pathogens of national and trade significance. The key outcome for each disease will be an estimate of pool-level diagnostic sensitivity for the disease and applicable method*"**.

There are three major objectives outlined in the DAWR contract:

Objective 1: Optimisation of magnetic bead nucleic acid extraction.

Objective 2: Determination of pool-level sensitivity for (a) white spot syndrome virus (WSSV), (b) yellow head virus genotype 1 (YHV1) and variants and (c) *Megalocytivirus*.

Objective 3: Comparison of pool-sensitivities for processed tissue vs. tissue swabs for detection of WSSV.

A summary of the discussions is presented below.

Objective 1: Optimisation of magnetic bead nucleic acid extraction

Previous results have indicated that nucleic acid extraction from some animal tissues using magnetic beads is not as efficient as using column extraction, leading to a decreased sensitivity of the magnetic bead method compared with the column method. Discussion with the molecular reagent vendor has led to identification of potential solutions viz. alternative reagents for use with the magnetic beads. These reagents will be used in an attempt to optimise magnetic bead extraction in an effort to increase the sensitivity of this method to be at least as sensitive as the column extraction method.

In order to achieve equivalency between magnetic bead and column nucleic acid extractions, various extraction kits (e.g. MagMax Viral RNA isolation kit, MagMax CORE kit) will be used and optimised by investigating the effect of sample volume, wash times, clarification solutions etc. on qPCR test performance (i.e. C_T values) using both extraction methods (i.e. magnetic bead and column).

Objective 2a: Determination of pool-level sensitivity for white spot syndrome virus (WSSV)

In Australia, a number of different tissues have been used previously as diagnostic samples by different jurisdictions when testing for the presence of WSSV in crustaceans viz. prawn tail muscle, prawn/crab gills, pleopods (as dictated by the OIE), as well as prawn tail muscle swabs.

The use of these various tissues was discussed and the various tissues have been included in the experimental design for determining WSSV pool-level sensitivity. To determine pool-level sensitivity data, artificially generated pools (tissue samples from 4 WSSV-negative and 1 WSSV-positive prawn) will be used in a highly controlled manner to produce robust data for analysis.

i. WSSV pool-level sensitivity - pools of 5 (muscle tissue)

The source material to be used for preparing experimental pools will be the retail prawns from DAWR testing phases 1, 2 and 3 (n=744) obtained following the SE Queensland WSSV outbreak. These prawns have been maintained frozen at -20 °C. The following steps to prepare the experimental material will be undertaken.

1) Muscle tissue sampling methodology development

To determine whether or not WSSV is distributed uniformly in muscle tissue in prawns with low-level infections, a sampling technique will be developed for obtaining samples of muscle that are consistent (by weight/volume) within and between prawns and when being sampled by different diagnosticians. For example, there are implements available that could be used to obtain muscle core or "punch" samples in an easy and consistent manner. Samples of muscle tissue will be taken along the length of known WSSV-infected prawns and analysed by WSSV-specific qPCR tests. Results will demonstrate the distribution of WSSV in the muscle tissue and provide information on the optimal site to be sampled.

2) WSSV test-negative retail prawns

Prawns (n=975) from the pools (n=195) that tested originally as negative will be re-tested individually. If positives are detected then WSSV detection uniformity throughout the tail muscle will be tested (as described above in (1)). The confirmed negative prawns will be used for creating the artificial pools for pool-level sensitivity testing.

3) WSSV test-positive retail prawns

To obtain individual WSSV-positive prawns with C_T values in the range of 25-40, individual prawns (n=~200) from positive pools (n=~40) will be tested. Thus, confirmed positive prawns (with C_T values in the range 25-40) will be identified for creating the artificial pools for pool-level sensitivity testing.

4) Experimentally created pools (includes clinical and sub-clinical C_T values)

Using prawns from (2) and (3) above, 40 pools of one positive and four negative muscle core/punches will be generated. In addition, 10 pools of five negative muscle core/punches will be interspersed between the "positive" pools prior to WSSV testing the experimental pools. These pools will be tested "blind" and pool-level diagnostic sensitivity vs individual-level sensitivity will be determined.

ii. WSSV pool-level sensitivity - pools of 5 (muscle swabbing)

Using the same animals as above, uniformity of WSSV detection in tail muscle by swabbing will be determined. In addition, these animals will be used for testing swabs individually and in pools of 5 (similar to the testing undertaken for the muscle tissue samples) to determine pool-level sensitivity using pooled swabs vs sensitivity using individual swabs. It should also be possible to determine whether or not sensitivity of muscle core/punches and swabs has been affected any differently.

Statistics and data analysis will be undertaken by recognised experts (e.g. Dr Axel Colling for MedCalc etc. and Dr Ian Gardner for Bayesian analysis).

Part of the analysis will also include determining the relative cost of testing samples from individual prawns vs. pooled samples (reagents and labour).

iii. WSSV pool-level sensitivity - pools of 5 (gills and pleopods)

Material that we do not have, i.e. whole prawns that are infected with WSSV by a natural route of infection, will need to be created. Such prawns will provide us with gills, pleopods and tails for analysis. We know from previous experiments that an inoculum with a C_T value of 32-36 for WSSV can be used

to infect prawns (by the i.m. route) and co-habited prawns will become infected. Sub-clinically infected animals can be collected at 3, 5 and 7 dpi (any remaining prawns will be kept until they die) and these will provide material with a range of C_T values.

a) Test 30 WSSV-exposed individual animals using all four sampling methods – gills, pleopods, muscle core/punches and swabbing:

   i) Sample a range of gills on both sides from individual animals

   ii) Sample pleopods (first, mid and last) on both sides of the tail

   iii) From the tail - sample muscle using both core/punches and swabs

   iv) Test for WSSV. Determine whether or not WSSV is distributed uniformly and detected by each sampling technique.

b) Generate pools of 5 gills and pleopods to test pool-level sensitivity (depends on results from different tissues in a). Determine which tissue is optimal. This will be a similar design to muscle core/punches and swabbing experiments as described above.

ADDITIONAL NOTES

Example Method (muscle tissue or gills/pleopods)

- Muscle core/punch or gills/pleopods taken from 5 individual animals and added to a bead-beating tube
- Tissue homogenized by bead-beating
- Magnetic bead extraction (spiked with T4 DNA bacteriophage)
- Nucleic acid tested neat by qPCR (with no or minimal inhibition)
- qPCR Tests: CSIRO WSSV (in duplicate), T4 (singlicate)

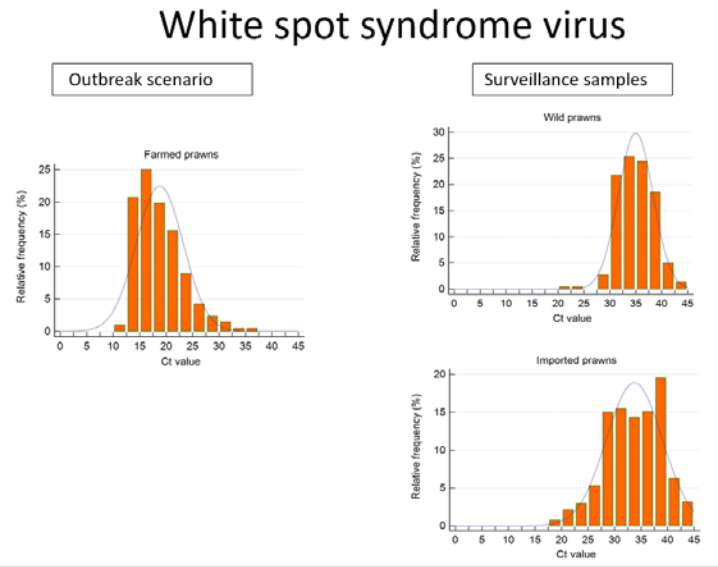
Example Method (muscle swab)

- Swab muscle from 5 individual animals. Add to collection tube.
- Collection tube is vortexed and incubated. An aliquot is taken and added to extraction solution.
- Magnetic bead extraction (spiked with T4 DNA bacteriophage)
- Nucleic acid tested neat by qPCR (with no or minimal inhibition)
- qPCR Tests: CSIRO WSSV (in duplicate), T4 (singlicate)

DSe using current methods has been calculated to be 79% (lower limit) from the WSSV draft manuscript (based on two populations imported/wild) in preparation. There it is calculated that 184 individuals from a population are to be tested in duplicate with one qPCR.

Pools are to be created from 5 individuals (1 positive and 4 negatives) to determine whether or not sensitivity and specificity are affected by pooling. For this study, 40 pools of 5 (total of 200 prawns) are to be tested. This number is based on previous DSe (79%) and numbers to be tested (184), as above.

Pools will be created using individual animals with a range of known C_T values. The frequency distribution of the C_T values will be based on the C_T value profile obtained for the imported and wild prawns visualized with MedCalc (in WSSV manuscript in preparation):



For example, for a distribution of C_T 25-30 (10%), C_T 30-35 (45%), C_T 35-40 (45%), we will need to create 4 pools using single animals with C_T values of 25-30, 17 pools using single animals with C_T values of 30-35 and 17 pools using single animals with C_T values of 35-40 and determine whether or not the frequency distribution of the pooled samples is the same as that for the individual prawns.

The new sampling method (muscle core/punch) is required to be easier than the current cuticular epithelium collection. This will reduce the current variability in the amount of tissue sampled from each animal. Therefore the selected sampling method needs to provide a consistent piece of muscle taken as core/punch from retail peeled prawn tails. The method needs to be repeatable and instruments are to be disposable. Options include syringe/needle, wide bore filter pipette tips, or other similar device. The instrument should have a graduation scale so that the amount of tissue sampled is easily repeatable. The amount of tissue will be informed by the level of T4 inhibition determined from various sized core/punch muscle samples (in pools of 5).

Other considerations

Swabbing

To save time should individuals be sampled for tissue at the same time they are swabbed as individuals and pools?

- Do multiple cuts of the tail swabbed return the same C_T from the same animal?
- Can you swab the same site multiple times on an individual and get similar results?

Generation of Pools

Negative animals

- 4 different negative individuals will be used to take a core/punch sample from for each pool (rather than four tissue samples from a single prawn)
- by using different animals each time to generate the negatives as part of the pool, greater variation in the sample (inhibitors, condition, origin etc.) will be generated that may affect results and will provide a better indication of the method's robustness.
- Therefore at least 160 negative animals will be required. Variation in prawns will be informed by the information that we have for the retail prawns:
    - country of origin (if provided)
    - marinated or not (would need to be noted upon thawing)
    - size (would need to be noted upon thawing)

In order to prevent cross-contamination, care with respect to the order that samples are taken will be required. For example, to create the pools, the four negative muscle core/punch samples into bead-beating tubes will be added in one safety cabinet. The tubes will be transferred to a different safety cabinet for the addition of the single positive muscle core/punch samples to each of the tubes.

Sampling from farmed prawns or wild prawns is likely to be from pleopods or gills:

- Consider clinical and subclinical
- Gather information of what sample (gills, pleopods, muscle swab or tissue) is optimal for test consistency.

Objective 2b: Determination of pool-level sensitivity for yellow head virus (YHV)

i. YHV1 pool-level sensitivity - pools of 5 (muscle tissue and swabbing)

Naturally YHV1-infected samples would be optimal source material for generating pools to determine pool-level sensitivity for YHV testing. Since YHV1 is exotic to Australia naturally infected samples are limited. Potential sources of naturally infected prawns include:

- Retail supermarket peeled prawn tails in pools of 5 from DAWR phase 1, 2 and 3 (n=744). YHV1 status is unknown and this would need to be tested using individual prawns.
- Sub-clinical YHV1 from China and Ecuador ethanol-fixed animals
- Experimentally infected animals and generation of preclinical animals by co-habitation (similar to that described for WSSV)

The experimental design will be similar to that described for WSSV i.e. create pools and test for YHV1 (includes clinical and sub-clinical C_T values):

i) Generate 40 pools of one positive and four negative muscle core/punches. Generate 10 pools of five negative muscle core/punches to be interspersed with the “positive” pools.

ii) Test pools for YHV1 and determine pool-level sensitivity vs. individual-level sensitivity

iii) Determine by swabbing uniformity of YHV1 detection in tail muscle

iv) Animals used for 50 pools of muscle tissue core/punches also swabbed and tested for YHV1

- determine if swabbing pool vs individual testing has been affected
- has sensitivity of muscle core/punches or swabbing been affected differently?

v) Statistics and data analysis

vi) Cost of testing individual prawns vs pool (including labour)

ii. YHV1 pool-level sensitivity - pools of 5 (gills and pleopods)

For source material, if naturally infected whole animals cannot be obtained, then experimentally infected animals will be used.

a) Test individual animals by all four sampling methods – gills, pleopods, muscle core/punches and swabbing

i) Animals sampled on different days should be considered. Gills should turn positive first.

ii) Sample a range of gills from individual animals on both sides

iii) Sample pleopods (first, mid or last) on tail on both sides

iv) Sample tail muscle core/punches and swab muscle

v) Test to determine whether or not YHV1 is distributed uniformly and detected by each sampling technique.

b) Generate pools of 5 gills and pleopods to test pool-level sensitivity (depends on results from different tissues in 1) - what tissue is optimal? Similar design to muscle core/punches and swabbing.

ADDITIONAL NOTES

Retail supermarket peeled prawn tails in pools of 5 may contain YHV1 positives. Screen pooled NA from the 744 retail prawns using CSIRO YHV RT-qPCR and/or AFDL YHV1 RT-qPCR to identify YHV-positive samples. Screen ~200 (3 plates of 88) at a time until sufficient positive prawns are identified. Preferentially test prawns from Thailand, China and Vietnam, countries which are known to have YHV1.

If sufficient naturally infected samples cannot be obtained, then material will need to be generated experimentally via cohabitation exposures to generate pre-clinically infected animals.

Since YHV1 kills prawns rapidly an inoculum with a relatively high C_T will need to be used. Haemolymph from YHV1-infected prawns (ex Thailand) will be the source of the inoculum. Inject (i.m.) 25 animals and use upto 40-50 co-habiting animals/tank (3 co-habitation tanks to be set-up).

20 animals collected on days 1, 2, 3, 4, 5 and 6 dpi. Whole animals (pre-clinical) will be euthanised, placed individually into plastic bags and stored at -80 °C. Dead animals (clinical) will also be collected in the same way.

Objective 2c: Determination of pool-level sensitivity for *Megalocytivirus*

Megalocytivirus *pool-level sensitivity - pools of 5*

Naturally *Megalocytivirus*-infected samples would be the optimal source material for generating pools to determine pool-level sensitivity for *Megalocytivirus* testing. Potential sources of naturally infected fish include:

- Retail animals - but new regulations may have eliminated this as a source
- Left-over positive samples from the ornamental fish reform project – low number positives 5-10%.
- Arrange to import fish directly to AAHL from overseas regions known to have positive fish. Probably require 500 fish if 10% prevalence of sub-clinical infection to result in 50 positives. (N.B. will likely require special import but may not require permit associated with environment department)

To obtain negative fish may require importing lower numbers from a reliably negative source.

If naturally infected animals cannot be sourced, it will be necessary to use preclinical samples generated experimentally:

Previously, we obtained an ISKNV-infected imported Angelfish sample from Dr Terry Miller (Agriculture WA) and were able to isolate infectious virus using the SKF-9 cell line (Kawato *et al*. 2017). Thus we will be able to use cell culture-derived material as a source of virus for experimental infection of animals (two species, most likely Platys and Gouramis) by bath immersion. This material will be used to generate the artificial pools.

Experimental infections

Previous studies have demonstrated that sub-clinically infected ornamental fish species have been detected with C_T values in the range mid-20s to mid-40s. Therefore the aim would be to generate pools that reflect the frequency distribution of individual test results (C_T values) including those obtained for the sub-clinically infected fish:

1) Create pools and test for *Megalocytivirus* (includes clinical and sub-clinical C_T values)

   i) Generate 40 pools of one positive and four negative homogenates (target organs or viscera) and 10 pools of five negative homogenates

ii) Test pools (blind) for *Megalocytivirus*: determine pool-level sensitivity and compare with individual-level sensitivity.

iii) Statistics and data analysis

vi) Cost analysis of testing individual prawns vs pool (including labour)

2) If experimental infections are performed there is the opportunity for additional data to be generated and considered, e.g. titrations of virus, two fish species challenged, re-isolation of the virus, injecting sub-clinical homogenates into susceptible species etc.

ADDITIONAL NOTES

Positive and negative fish from reliable source for generation of pools and for experimental infections?

Which species? Cichlids, Poeciliids and Pterophyllum, Platys and Gouramis (check FRDC report 2014/001)

Experimental infections

Titration of virus and 10-fold dilute and expose experimental groups of fish the various doses:

4 doses and negative controls (120/dose) – require ~780 fish – if 50 pre-sampled to confirm negative

Dose titration (10-fold dilutions; 4 doses + uninfected controls). Expose fish in 10-20 litres for 1 hour and then transfer to 2 X 100 L aquaria. Water temp = 25 C. Uninfected controls in a separate room.

Take samples every second day for 10 d (12 from each group sampled per day) – max. 20 days depending on mortality rate.

Potentially run experiment longer than 10 d if no deaths to get a better estimate of pre-clinical status.

Clinical disease expected from day 5 onwards for highly susceptible host species.

Platys and perhaps guppies or gouramis.

If 50 culled and tested for *Megalocytivirus* first prior to experimental infection. If unknown source population more would need to be tested.

Sample collection and processing

Sample viscera from infected fish and negative controls on day of collection, bead beat, test and keep homogenates (to use later for pooling).

Viscera to contain kidney, liver and spleen but will also contain other organs similar to the testing done during OFIR project.

Sampling every second day for the first ten days.

On day of sampling in LAF take-out viscera and place in ethanol (80% v/v) for each fish. Take out of LAF and bead-beat later individually. Take homogenate for testing of individual.

Pool generation

Pooling to take 40 positive individuals with selection: only fish without clinical signs (pre-clinical) to be used for pools

Pooling to be performed similar to WSSV and YHV1 protocols: equal volumes of homogenate to create pools.

Results

I. Objectives 1-3

For objectives 1-3, a total of 556 studies underwent initial screening. Of these, 262 underwent full-paper analysis. Most studies used qPCR, histopathology and *in situ* hybridisation for diagnosis. For finfish some studies used virus isolation in cell culture followed by virus identification using ELISA and/or IFAT. The results of this analysis are encompassed in the publication:

Laurin E, Thakur KK, Gardner IA, Hick P, Moody NJG, Crane MSJ, Ernst I. 2018. Design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. *J Fish Dis* **41:** 729-749.

Objectives 1-3

1. Evaluate (a) design criteria reported for diagnostic studies of OIE-listed diseases of finfish, molluscs and crustaceans and (b) test design and accuracy recommendations from OIE, peer-reviewed literature in aquatic animal health and other sources (e.g. aquatic animal health experts).

2. Develop a list of gaps or inconsistencies in current design criteria and reporting for OIE-listed diseases in aquatic species, based on collected recommendations from Objective 1.

3. Compile consensus-based standards in an applicable format for diagnostic studies in finfish, molluscs and crustaceans for use by research journals and laboratories.

The manuscript describes recommended design standards for consideration in future field or experimental studies that address purpose, target condition, sample selection, index test, latent class analysis, analytical methods, and interpretation of estimates of diagnostic sensitivity and specificity.

II. Objective 4

With respect to Objective 4, a summary of the experimental protocols is provided for each of the DAWR contract objectives.

Objective 4

Develop experimental protocols for the determination of pool-level diagnostic sensitivity for the qPCR detection of sub-clinical infections involving specific pathogens (WSSV, YHV, *Megalocytivirus*), with input from an expert aquatic epidemiologist.

DAWR contract Objective 1: Optimisation of magnetic bead nucleic acid extraction

In order to achieve equivalency between magnetic bead and column nucleic acid extractions, various extraction kits (e.g. MagMax Viral RNA isolation kit, MagMax CORE kit) will be used and optimised by investigating the effect of sample volume, wash times, clarification solutions etc. on qPCR test performance (i.e. C_T values) using both extraction methods (i.e. magnetic bead and column).

DAWR contract Objective 2a: Determination of pool-level sensitivity for white spot syndrome virus (WSSV)

To determine pool-level sensitivity data, artificially generated pools (tissue samples from 4 WSSV-negative and 1 WSSV-positive prawn) will be used in a highly controlled manner to produce robust data for analysis.

i. WSSV pool-level sensitivity - pools of 5 (muscle tissue)

The source material to be used for preparing experimental pools will be the retail prawns from DAWR testing phases 1, 2 and 3 (n=744) obtained following the SE Queensland WSSV outbreak. Firstly, the distribution of WSSV in the muscle tissue will be determined and used to provide information on the optimal site to be sampled. Confirmed WSSV-negative and positive retail prawns will be used to generate 40 pools of one positive and four negative muscle core/punches. In addition, 10 pools of five negative muscle core/punches will be interspersed between the "positive" pools prior to WSSV testing the experimental pools. These pools will be tested "blind" and pool-level diagnostic sensitivity vs individual-level sensitivity will be determined.

ii. WSSV pool-level sensitivity - pools of 5 (muscle swabbing)

Using the same animals as above, uniformity of WSSV detection in tail muscle by swabbing will be determined. In addition, these animals will be used for testing swabs individually and in pools of 5 (similar to the testing undertaken for the muscle tissue samples) to determine pool-level sensitivity using pooled swabs vs sensitivity using individual swabs. It should also be possible to determine whether or not sensitivity of muscle core/punches and swabs has been affected any differently.

iii. WSSV pool-level sensitivity - pools of 5 (gills and pleopods)

Material not available, i.e. whole prawns that are infected with WSSV by a natural route of infection, will be created. Such prawns will provide us with gills, pleopods and tails for analysis. An inoculum with a C_T value of 32-36 for WSSV will be used to infect prawns (by the i.m. route) which will be used to infect co-habited prawns (natural route of infection). Sub-clinically infected animals can be collected and these will provide material with a range of C_T values which will be used to generate pools of 5 gills and pleopods to test pool-level sensitivity (depends on results from different tissues in

DSe using current methods has been calculated to be 79% (lower limit) from the WSSV draft manuscript (based on two populations imported/wild) in preparation. There it is calculated that 184 individuals from a population are to be tested in duplicate with one qPCR.

Pools are to be created from 5 individuals (1 positive and 4 negatives) to determine whether or not sensitivity and specificity are affected by pooling. For this study, 40 pools of 5 (total of 200 prawns) are to be tested. This number is based on previous DSe (79%) and numbers to be tested (184), as above.

Pools will be created using individual animals with a range of known C_T values. The frequency distribution of the C_T values will be based on the C_T value profile obtained for the imported and wild prawns visualized with MedCalc.

Objective 2b: Determination of pool-level sensitivity for yellow head virus (YHV)

i. YHV1 pool-level sensitivity - pools of 5 (muscle tissue and swabbing)

Naturally YHV1-infected samples would be optimal source material for generating pools to determine pool-level sensitivity for YHV testing. Since YHV1 is exotic to Australia naturally infected samples are limited. Potential sources of naturally infected prawns include:

- Retail supermarket peeled prawn tails in pools of 5 from DAWR phase 1, 2 and 3 (n=744). YHV1 status is unknown and this would need to be tested using individual prawns.
- Sub-clinical YHV1 from China and Ecuador ethanol-fixed animals
- Experimentally infected animals and generation of preclinical animals by co-habitation (similar to that described for WSSV)

The experimental design will be similar to that described for WSSV i.e. create pools and test for YHV1 (includes clinical and sub-clinical C_T values)

ii. YHV1 pool-level sensitivity - pools of 5 (gills and pleopods)

For source material, if naturally infected whole animals cannot be obtained, then experimentally infected animals will be used, similar to the WSSV experiments. Four samples, gills, pleopods, muscle core/punches and swabbing will be taken and tested to determine whether or not YHV1 is distributed uniformly and detected by each sampling technique. Pools of 5 gills and pleopods will be generated to test pool-level sensitivity.

Objective 2c: Determination of pool-level sensitivity for *Megalocytivirus*

Megalocytivirus *pool-level sensitivity - pools of 5*

Naturally *Megalocytivirus*-infected samples would be the optimal source material for generating pools to determine pool-level sensitivity for *Megalocytivirus* testing. Potential sources of naturally infected fish include retail animals (but new regulations may have eliminated this as a source), left-over positive samples from the ornamental fish reform project (but there are low number of positives). Alternatively, import fish directly to AAHL from overseas regions known to have positive fish (N.B. will likely require special import but may not require permit associated with environment department). To obtain negative fish may require importing lower numbers from a reliably negative source.

If naturally infected animals cannot be sourced, it will be necessary to use preclinical samples generated experimentally and then this material will be used to generate the artificial pools.

Objective 3: Comparison of pool-sensitivities for processed tissue vs. tissue swabs for detection of WSSV.

Analysis will be undertaken by expert aquatic epidemiologists (Axel Colling and Ian Gardner).

Based on the discussions held during Workshop III, the project team is well-prepared to undertake experimental studies to address the Commonwealth of Australia (DAWR) Contract 78260: "*To develop and validate fit-for-purpose levels of pooling for surveillance of aquatic animal pathogens of national and trade significance. The key outcome for each disease will be an estimate of pool-level diagnostic sensitivity for the disease and applicable method*".

Implications

Important metrics for consideration at the design phase of diagnostic accuracy studies (DAS), study purpose, targeted disease state (clinically diseased vs infected but apparently healthy), selection of appropriate samples and specimens, laboratory analytical methods, statistical methods and data interpretation, have been identified and published. Following standards for designing DAS will assist in appropriate test selection for specific testing purposes and minimize the risk of reporting biased estimates of diagnostic sensitivity and specificity.

With respect to pool-level sensitivity, using the developed protocols in DAS will ensure that any effect of pooling samples for the specified disease agents and hosts on diagnostic sensitivity will be clearly defined.

If the principles outlined in this project are followed for all diagnostic accuracy studies, diagnosticians, regulators, industry stakeholders and trading partners will have enhanced confidence in the accuracy of the diagnostic tests developed and assessed for use in the detection and identification of aquatic animal pathogens, including during surveillance activities.

Recommendations

Lack of quality standards makes it difficult to assess the appropriateness of diagnostic tests for a given purpose. Standardisation of the design stage of studies aimed at the development/assessment of diagnostic tests for aquatic animal diseases is recommended, taking into account a range of factors such as the purpose of the test, the target population, disease status, environmental conditions of the study, sample selection, experimental challenge design, analytical methods and interpretation.

It is recommended that the principles outlined in this project are followed for all diagnostic accuracy studies.

Further development

Proposals for future diagnostic accuracy studies should include a description of how the principles outlined in this project are to be addressed. The project team are continuing to collaborate with Ian Gardner on the issue of pool-level sensitivity and validation of diagnostic tests using test results from pooled samples.

Extension and Adoption

1. The experimental protocols developed as part of this project for determining the pool-level sensitivity for WSSV, YHV and *Megalocytivirus* will be utilised in the project to deliver against the Commonwealth of Australia (DAWR) Contract 78260: "*To develop and validate fit-for-purpose levels of pooling for surveillance of aquatic animal pathogens of national and trade significance. The key outcome for each disease will be an estimate of pool-level diagnostic sensitivity for the disease and applicable method*".

2. By publishing the findings of this project with respect to design standards for both experimental and field studies aimed at developing/assessing the accuracy of diagnostic tests for infectious diseases of aquatic animals in a peer-reviewed journal we have ensured the widest possible audience is provided the opportunity to review and hopefully adopt this output of the project.

3. Outputs from this project include:

   - Full paper published in a peer-reviewed scientific journal:

     Laurin E, Thakur KK, Gardner IA, Hick P, Moody NJG, Crane MSJ, Ernst I. 2018. Design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. *J Fish Dis* **41:** 729-749.

   - Presentations made at the 18th European Association of Fish Pathologists International Conference on Diseases of Fish and Shellfish, Belfast UK, 4-8 September 2017:

     Gardner I, Laurin E, Hick P, Crane M, Moody N, Ernst I, Thakur K. 2017. Design standards for experimental and field studies to evaluate diagnostic accuracy of tests for infectious diseases in aquatic animals. Abstract 115-O.

     Laurin E, Gardner I, Moody N, Crane M, Hick P, Ernst I. 2017. To pool or not to pool? Guidelines for pooling specimens for different diagnostic end purposes in aquatic animals. Abstract 116-O.

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Appendices

Appendix 1. List of researchers and project staff

Appendix 2. Workshops 1-3 Agenda

Appendix 1. Participant list

Name	Institute
Mark Crane	CSIRO-AAHL Fish Diseases Laboratory, Geelong, Australia
Nick Moody	CSIRO-AAHL Fish Diseases Laboratory, Geelong, Australia
Peter Mohr	CSIRO-AAHL Fish Diseases Laboratory, Geelong, Australia
Axel Colling	CSIRO-AAHL Veterinary Investigation & Epidemiology, Geelong, Australia
Ian Gardner	Atlantic Veterinary College, University of Prince Edward Island, Canada
Emilie Laurin	Atlantic Veterinary College, University of Prince Edward Island, Canada
Yuko Hood	Department of Agriculture & Water Resources, Canberra, Australia
Ingo Ernst	Department of Agriculture & Water Resources, Canberra, Australia
Paul Hick	University of Sydney, Camden, New South Wales, Australia

Appendix 2. Workshop Agenda

I. Canberra Workshop I Agenda

Tuesday 29 March 2016

11:00 Welcome and introductions (Ian and Ingo, and all participants)

National and international context (Ingo)

11:30 Overview of purposes of meeting and proposed outcomes (Ian – main points below and Ian will be responsible for meeting report)

A. Design standards for diagnostic studies (in the context of fitness for purpose)

1) Develop outline and key points to be included in paper - goal is to publish in a peer-reviewed journal
2) Discussion of key points for inclusion – collectively and in break-out groups during meeting
3) Allocate and agree to writing assignments
4) Discussion of names of other colleagues to include as necessary
5) Timeline for remainder of project including a draft of a manuscript
6) List of action items and responsibilities post meeting

B. Pooling guidelines (in the context of fitness for purpose)

1) Discuss literature review findings and diseases of most interest in Australia and internationally
2) Discuss and agree on next steps
3) Timeline for completion of project
4) List of action items and responsibilities post meeting

13:30 Updates on design standards to inform discussions later that day and on Wednesday morning

1) Progress on literature review (Emilie and Ian) including
    i. studies identified for possible use as examples
    ii. writing
2) Experimental challenge designs (Paul to lead, with additional comments at the end from Nick, Mark, Ingo) – how well do published studies mimic natural infection? Initial ideas on "must haves" and "nice to haves" in the design; how prescriptive should we be and any additional thoughts; good example studies, if they exist (note: the difficulty may be differentiating reporting from design quality
3) Field designs – strengths and weaknesses of different approaches, biases; what should be emphasized or added to points made in Nielsen *et al*. (2011), including adding something on acute diseases; published papers with good designs for possible use (Charles to lead with additional comments from Ian and Emilie)

15:00 Breakout discussion in experimental design and field design groups

16:00 Reports and discussion of entire group

17:00 Wrap and action items from day, if any

Wednesday 30 March 2016

9:00 Review/summary from Day 1 (All)

9:30 Continue unfinished discussion of design standards from Tuesday

13:00 Pooling project - suggested items for presentation are below (to discuss on Tuesday or by e-mail in advance of meeting)

- National and international context (Ingo)
- Summary of findings from published papers and OIE chapters (Emilie)
- Discussion of findings (All)
- Additional perspectives from published or unpublished studies (Nick and Mark, Paul, Charles)
- Next steps including discussion of what it would take to make this work publishable (All)

16:30 Wrap-up and action items

17:00 Meeting closes, depart for airport

II. Geelong Workshop II Agenda

Thursday 16 March 2017

9:00 Brief update on progress (Ian and Emilie)

Discussion to include agreement on authorship of publications and the target journal (All)

10.00 Empirical data: experimental trial and/or theoretical/simulated data? Ideas on what would be available and when? Goals on what we want to get out of it to support the guidelines we are proposing in the manuscript? (All)

11.00 Discussion on purposes - surveillance and/or presumptive/confirmatory diagnosis, use of pools for estimation of prevalence (All)

13.00 Consensus discussions on what guidelines are "must haves" and then make a list of "nice-to-haves". For example, the study characteristics (e.g. applicable purposes for using pooling methods, index tests, case definition, source populations, pathology of disease, prevalence, etc.), *a priori* pool characteristics (e.g., number/pool, number of pools, TUE's DSe/DSp, etc.), sample/specimen characteristics (e.g., collection type and method, tissue types, representative tissues, storing, presence of inhibitors or cross-contamination, etc), specimen/sample prep methods (e.g., homogenization and concentration and extraction methods and timing with regards to pooling, comparable methods for individual vs. pooled specimens, etc.), diagnostic interpretation and statistical analyses methods (e.g., comparing individual to pooled results, tissue/sample type/etc-specific effects, dilution and other biased effects due to pooling, extrapolation to target pop from pooled results depending on load and prevalence, etc.) (All)

17.00 End of day 1

Friday 16 March 2017

9:00 Brief review of previous day (Ian)

9:30 Discussion on specific disease agents to be included. Access to data already available. Experimental work to be undertaken to generate new data that can value-add. (All)

10:30 Assigning tasks: Preliminary timeline on who's doing what parts and timeline for completion and submission of a paper. (All)

11:00 Discussion of on-line tools (http://epitools.ausvet.com.au/content.php?page=home). Should any of the pooled testing sheets be included? (All)

13:00 End of day 2

III. Geelong Workshop III Agenda

Friday June 22 2018

09.00 Welcome and confirmation of agenda

Project team: Axel Colling, Ian Gardner, Mark Crane, Nick Moody, Peter Mohr (Yuko Hood will join us on Tuesday)

09.10 Purpose of meeting and proposed outcomes

To discuss experimental methodology to determine pool-level diagnostic sensitivity leading to development of experimental design for undertaking DAWR Pool-level sensitivity project.

Ian to lead: Definition of *pool-level diagnostic sensitivity* (detection of sub-clinical infections); methods to determine *pool-level diagnostic sensitivity* for detection of sub-clinical infections

10.30 Coffee break

11.00 2018 DAWR Pool-level sensitivity project

Aim: To develop and validate fit-for-purpose levels of pooling for surveillance of aquatic animal pathogens of national and trade significance. The key outcome for each disease will be an estimate of pool-level diagnostic sensitivity for the disease and applicable method.

Objective 1: Optimisation of magnetic bead nucleic acid extraction.

Objective 2: Determine pool-level (pools of 5) sensitivity for (a) white spot syndrome virus (WSSV); (b) yellow head virus genotype 1 (YHV1) and variants; (c) *Megalocytivirus*.

Objective 3: Comparison of pool-sensitivities for processed tissue vs. tissue swabs for detection of WSSV.

12.00 General discussion: Field samples vs experimental infection samples

12.30 Lunch

13.30 Current resources

Peter: - Stocktake of field samples (WSSV; YHV1; *Megalocytivirus*)
- Stocktake of experimental samples

14.30 Other requirements: Need for further field samples and/or experimental infections

15.30 Tea break

16.00 Planned DAWR Project Activities

Objective 1: Optimisation of magnetic bead nucleic acid extraction.

Objective 2: Determine pool-level (pools of 5) sensitivity for (a) white spot syndrome virus (WSSV); (b) yellow head virus genotype 1 (YHV1) and variants; (c) *Megalocytivirus*.

Objective 3: Comparison of pool-sensitivities for processed tissue vs. tissue swabs for detection of WSSV.

Activity description	Estimated completion date
High-throughput nucleic acid extraction using magnetic beads optimised.	30/09/2018
Individual prawns tested for white spot syndrome virus (WSSV) to determine samples to be used in pooling study. Pooling study planned and pools (of five) tested.	31/12/2018
Data analysed and pool-level (pools of 5) sensitivity determined for WSSV.	31/05/2019
Progress Report 1 submitted to the satisfaction of the DAWR.	30/06/2019
Individual prawns tested for yellow head virus genotype 1 (YHV1) to determine samples to be used in pooling study. Pooling study planned and pools (of five) tested.	30/11/2019
Data analysed and pool-level (pools of 5) sensitivity determined for YHV1.	31/03/2020
Progress report submitted to the satisfaction of the DAWR.	30/04/2020
Individual fish tested for *Megalocytivirus* to determine samples to be used in pooling study. Pooling study planned and pools (of five) tested.	31/10/2020
Data analysed and pool-level (pools of 5) sensitivity determined for *Megalocytivirus*.	28/02/2021
Progress report submitted to the satisfaction of the DAWR.	31/03/2021
Draft final report submitted to the DAWR for comment and review.	30/04/2021
Final report submitted to the satisfaction of the DAWR.	30/06/2021

17.00 Close Day 1

Monday June 25 2018

09.30 Review of Day 1

10.00 Planned DAWR Project Activities revisited

10.45 Coffee break

11.00 Pooling guidelines and proposed experiments

(1) WSSV
(2) YHV1
(3) Megalocytivirus

12.30 Lunch

13.30 Pooling guidelines and proposed experiments continued

15.30 Tea break

16.00 Review of DAWR Project Milestones

2018 DAWR Pool-level Sensitivity Project: Milestone Reports

Milestone	Detailed Description	Due Date
Progress Report 1	A summary of results from: • optimisation of high-throughput nucleic acid extraction using magnetic beads • individual prawns tested for WSSV • data analysis of pooling study and pool-level (pools of 5) sensitivity for WSSV. As well as an explanation of why any milestone or activity specified in this contract for the period covered by the report was not conducted or achieved, and how they are to be conducted or achieved.	30-Jun-19
Progress Report 2	A summary of results from: • individual prawns tested for YHV1 • data analysis of pooling study and pool-level (pools of 5) sensitivity for YHV. As well as an explanation of why any milestone or activity specified in this contract for the period covered by the report was not conducted or achieved, and how they are to be conducted or achieved.	30-Apr-20
Progress Report 3	A summary of results from: • individual fish tested for *Megalocytivirus* • data analysis of pooling study and pool-level (pools of 5) sensitivity for *Megalocytivirus* As well as an explanation of why any milestone or activity specified in this contract for the period covered by the report was not conducted or achieved, and how they are to be conducted or achieved.	31-Mar-21
Final Report	The final report should contain the information required in the progress reports, together with: • a summary of activities over the whole term of the project • a full reconciliation against the budget, a statement of the balance of the bank account including any interest earned • a statement as to whether the project was carried out in accordance with the objectives, milestones, and activities.	30-Jun-21

17.00 Close Day 2

Tuesday June 26 2018

09.30 Review of discussions to-date

10.00 DAWR input (Yuko Hood)

10.30 Coffee break

11.00 Planned DAWR Project Activities revisited

12.00 Agreement on workshop outputs

12.30 Lunch

13.00 AOB

13.30 Workshop close