COMPLETED PROJECT CASE STUDY

FULL R&D: PRE-EMPTIVE AND NON-INVASIVE PATHOGEN DIAGNOSTICS TO PREVENT THE SPREAD OF SHELLFISH DISEASE

PARTNERS

University of Edinburgh – The Roslin Institute

PROJECT LEADS

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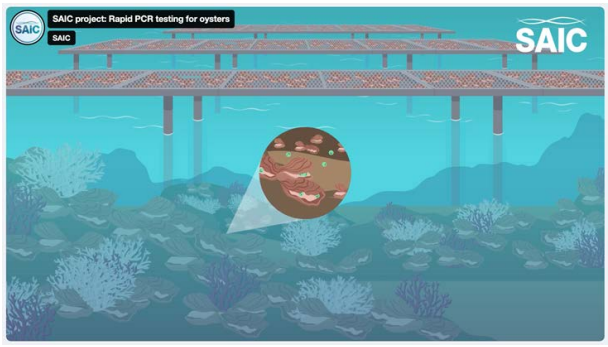
FUNDERS

Seafood Innovation Fund (SIF), with support from SAIC.

The UK Seafood Innovation Fund supports new ideas to deliver cutting-edge technology and innovation to the UK's fishing, aquaculture and seafood industries. Administered by the Centre for Environment, Fisheries and Aquaculture Science (Cefas) on behalf of the Department for Environment, Food and Rural Affairs (Defra), the programme encourages sustainable and innovative ideas to bring about seafood security, new partnerships across seafood and technology sectors, and to contribute to strong evidence-based management.

Watch this short animation to get an overview of the project:



BACKGROUND

Disease is the single biggest issue facing oyster aquaculture and restoration. Regulation and monitoring aim to classify the health of a particular area by assignation of pathogen presence or absence for key listed pathogen and host species to prevent movement of disease by prohibiting movement of vector animals.

Measures to stop the spread of oyster diseases in the UK rely entirely on the prevention of animal movement from disease positive to disease free sites. However, most current centralised diagnostic tests are reactive, taking place once a disease is already present. These measures are not entirely successful, resulting in gradual spread of pathogens across the UK.

There are a few options currently available for farmers to take proactive measures in pathogen control. It is possible to survey stock before transport by taking a subsample of animals and sending these to a professional or government organisation for analysis. However, this is costly, requiring trained staff and testing over an extended period of time, and only small numbers of animals are tested.

In direct response to requests from shellfish farmers and restoration practitioners, the previous feasibility project developed and tested protocols for pre-emptive pathogen diagnostics, combining a portable qPCR machine and field DNA extraction protocols for two common pathogens: oyster herpes virus (OsHV-1) and *Bonamia ostreae*.

OsHV-1 worked well in the lab, but the *Bonamia* assays were also thoroughly tested in the field and demonstrated high sensitivity throughout. Using an entirely field-based system, it was possible to consistently detect one diseased oyster in ten. It was suggested that a lower sensitivity could be possible.

The innovative pathogen detection system developed uses three distinct components to advance disease management and allow testing of batches: Existing tanks where shellfish are held overnight after harvest can be used as incubation facilities, after which the faeces and pseudo-faeces or water are sampled for DNA. Diagnostic assays are then run with the Biomeme qPCR system, which is able to detect pathogens and/or non-native species before they are moved onto other sites.

AIMS

The key aim of this project was to further validate and demonstrate the potential of the portable diagnostic process, which is suitable for use by shellfish farmers and ecological restoration practitioners. This included testing the system at field sites across the UK, including new assays, identifying the limits of detection, and describing sensitivities in typical shellfish quarantine scenarios, as well as developing simple user protocols and videos.

The development and use of a proactive testing system will benefit shellfish growers tremendously. Tube worm casts, while benign in terms of mussel quality, are difficult to remove and can interfere with packaging and presentation. Equally, Scotland has retained a disease-free status for oyster herpes virus, which causes losses of young shellfish. With improved detection methods, we would continue to seek to sustain this position, giving us advantages over shellfish production in surrounding countries. The industry is pleased to support this further development of techniques that will support our climate change resilience in the coming years.

Dr Nick Lake, CEO of the Association of Scottish Shellfish Growers

EXPERIMENTAL STUDY

This full R&D project builds on foundations developed in the initial feasibility study, which demonstrated the applicability of assays for *B. ostreae*, OsHV-1, *Crassostrea gigas* and *Ostrea edulis*, and is a natural extension and expansion of the previous work.

Initially, technical validation was performed, trialling DNA extraction of known positive material using the field-based M1 DNA extraction cartridge and running the assays on the Biomeme system. A new assay for B. ostreae was tested and the previous assays for *Crassostrea gigas* and *Ostrea edulis* were re-tested. Likewise, three new PCR assays were tested: *Vibrio aestuarianus*, the invasive non-native species *Didemnum vexillum*, and the polychaete tubeworm (*Pomatoceros spp*.).

Successful tests were accomplished for *V. aestuarianus, Vibrio splendidus, B. ostreae,* (utilising new primers), *Crassostrea gigas,* and *Ostrea edulis.* However, the DNA extraction system was not able to successfully extract useable DNA from *D. vexcillum.* The reasons for this are unknown, but are likely to be linked to the season during which samples were taken. It may be more appropriate to sample during early phase of growth, when there is more live material, rather than towards the end when tissues and DNA may be degraded. This resulted in the team moving away from working with *D. vexcillum* and instead to further study *V. splendidus,* as a more likely candidate for achieving successful results during a one-year project.

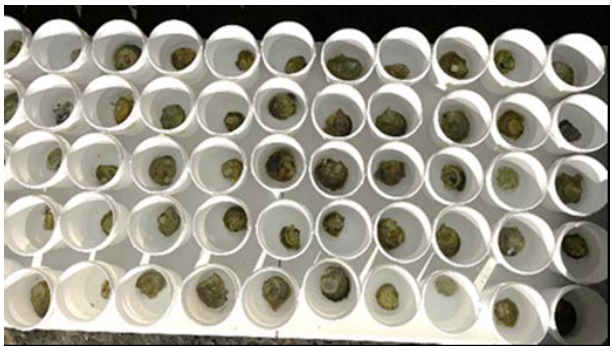
To define the minimum number of copies of DNA each qPCR assay could reliably measure in a standard laboratory reaction, the PGEM-T Easy Vector System (Promega, UK) was used to generate 18S ribosomal RNA gene from *B. ostreae* plasmids and these genes were cloned for validation through sequencing.

The qPCR assays were carried out in triplicate using the Applied Biosystems 7500 qPCR machine from Agilent technologies. The lowest concentration of DNA that can be detected with a Ct of 33.5 in all replicates was three copies per reaction. However, when amplifying concentrations below one copy, results become less reliable. As such, the limit of detection was set at 35 cycles and when amplification occurs with a Ct value below 35, it was suggested that confirmation of results was achieved.

The next step was to define the relative limit of detection of a Biomeme qPCR, with both laboratory and Biomeme M1 DNA extraction, compared to the standard laboratory analysis. To this end, sediment samples from West Loch Tarbert, which were confirmed to be free of *B. ostreae*, were used. DNA was extracted using an M1 DNA extraction kit according to the manufacturer's protocol, resulting in 1ml of DNA solution, from which a subsample was used in a PCR with all reactions carried out in triplicates.

To define the number of disease-positive oysters that can be detected in a quarantine system as a relationship to the standard amount of pathogen DNA shed per oyster, the new Biomeme-based method that had been developed was compared to histopathology and qPCR of gills and mantle.

To achieve this, following 16h incubation of 228 oysters in individual buckets (as seen in the picture below) the sediment remaining at the bottom of each bucket was collected into tubes.



Individual oysters incubating

The tubes were allowed to settle for five minutes before the excess supernatant was removed, leaving the sediment in the tube. DNA was extracted from this sediment and filtered. Following filtration, the sediment was processed through the Biomeme M1 extraction cartridge. Samples were pooled in groups and run on-site on the Biomeme qPCR machine against the three probe-based assays to assess for presence of the pathogen *B. ostreae.*

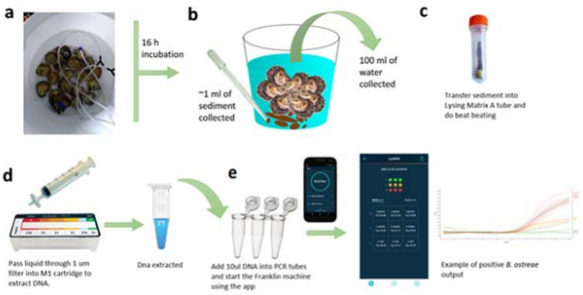
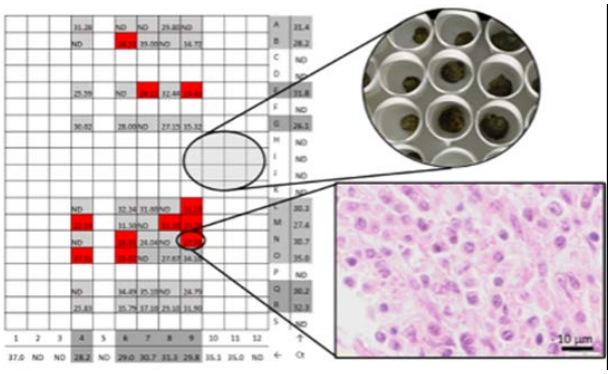


Diagram showing the process

Edulis DNA detection was performed to check the quality of the DNA extraction and a technical internal positive control (IPC) assay was undertaken to identify any amplification inhibition.

Pools that had a positive result were cross-checked and any oysters that intersected were then sampled for gill and mantle qPCR and histopathology by an accredited laboratory, as shown below.



Histopathology vs field-based qPCR

Eleven animals were diagnosed as positive for *Bonamia* by histopathology, ten mild (grade 2) and one very mild (grade 1). A further 16 samples showed some haemocyte infiltration in the tissues, which can be indicative of an infection, but no direct observation of *Bonamia.* Tissue qPCR detected *B. ostreae* in 35 of the 50 samples. Six of these samples were tentative positives, as their CT values were above 35, which is the cut-off for reliable detection. Of the additional 22 positive cases, eight showed haemocyte infiltration by histopathology but with no observation of *Bonamia* particles.

Individual sediment samples were also tested in the laboratory and the data suggested that qPCR of sediment identified *B. ostreae* in threefold more samples, in the field-run tests, than the histopathology. Hence showing that this methodology provides considerably higher sensitivity than traditional methods, and that it is as sensitive as gill and mantle qPCR at detecting infection in a population.

It has been observed that there is no standard amount of DNA shed per oyster, possibly due to the varying amount of faeces shed by each animal, which requires further study. However, the research was able to identify a mean amount shed and calculate a suggested power of detection based on this. A single infected animal has an average faeces Ct of 30. Power calculations based on limited data suggest an infection level of ~2% by qPCR that could be reliably detected when working with ~150 animals. Current histopathology methods also detect a 2% positive rate with 95% confidence. However, due to the lower overall sensitivity of histopathology on individuals, this 2% infection rate would be equivalent to ~6% by faeces qPCR. As such, sediment PCR is more sensitive.

As part of the project, scientific standard operating procedures (SOPs) were written in Protocols.io format, and video SOPs were produced in collaboration with a professional video production company. These videos will be made available online, and via QR code printed and displayed on the Biomeme system packaging. All further SOPs will continue to be published on www.protocols.io with associated doi numbers.

With the support of the SOPs developed, the system was further field trialled by running assays at four aquaculture sites with scientist assistance. Sites included Loch Dunvegan (Skye), Ulva (Mull), and West Loch Tarbert (Kintyre), as well as Heriot Watt University. There was highly positive feedback on initial runs about the requirement to have a system like this in place. Some critical feedback included that the process was still too 'clunky', required too much prior knowledge, and that the equipment may be too large.

Likewise, assays were run at aquaculture sites without scientist assistance, including at Lochnell offshore farm (run by SAMS), Wester Ross (Maorach Beag), and Loch Creran (Maorach Beag). The level to which people were able to successfully run these assays on their own varied. All participants were able to incubate oysters in a suitable system, collect and store oyster faeces, and send the samples to a laboratory. Several participants were able to extract DNA, but only one person, who had previous scientific training, was able to run the qPCR without any assistance. The project team therefore plans, upon project completion, to further break down the protocol into smaller and easier phases. These can then be followed by users up to the point at which they feel comfortable.

OUTCOMES

The procedure developed for the detection of *B. ostreae* has been proven to be sensitive, specific, robust, rapid, and simple enough for non-scientists to use.

The project has also been successful in demonstrating this protocol and its applications to scientific and industry communities, especially in using the system on-site at shellfish aquaculture locations.

The key output of this project is the development and validation of a technique that can be used to diagnose the presence of *B. ostreae* without requiring destructive sampling of animals. The technique is cheaper, faster, and more sensitive than the current gold standards of histopathology. It can detect a 2% prevalence in a batch of 150 animals. The sensitivity can be increased by batch-testing smaller numbers and, notably, the technique is considerably more likely to detect the presence of *Bonamia* in an individual animal from an infected population than the histopathology methods

currently employed by regulators. This is the case even with individuals with a low level of infection. As such, the technique is scalable, and accuracy can be maintained at scale by sub-sampling. Additionally, the method can be adapted for use in various scenarios, with samples being analysed directly in the field, or samples being taken from a field situation and subsequently analysed in the laboratory.

The capability of the system to be used for detection of oyster-infecting *Vibrio* bacterial species, both in the laboratory and field, has also been demonstrated.

The system proved much more challenging to test for use with invasive biofouling species, and it is suggested that more work is required in this area. However, all indications are that the technique could be expanded well beyond the scope already achieved.

The project also created potential long-term collaborations and partnerships, such as with Xelect Ltd and the Scottish Association for Marine Science (SAMS) to process a set of samples. This collaboration has grown and helped develop a Memorandum of Understanding for collaboration between the University of Edinburgh and SAMS, with the expectation of submitting joint grant proposals in the near future.

Similarly, the project has been instrumental in opening channels of communication with the shellfish sector, including Loch Creran, SAMS, Maorach Beag and Lochnell farms, as well as directly link with the Association of Scottish Shellfish Growers. It is expected that this will lead to future collaborative, sector-relevant projects.

It is unlikely that the University of Edinburgh will grow significantly as an output of this project; however the project has allowed the University's initial venture into the space of remote diagnostics. This project will lead to future scientific funding applications over the next five years.

ADDITIONAL INFORMATION

The research was presented and demonstrated at the Association of Scottish Shellfish Growers (ASSG) Annual Meeting in October 2022. This platform provided the opportunity to demonstrate and advertise the *Bonamia* assay to an industry audience, with an opportunity to obtain guidance and advice directly from the sector.

The technique was also presented at the National Shellfisheries Association's annual international meeting, Baltimore, March 2023: "Proactive and non-invasive pathogen diagnostics to prevent the spread of *Bonamia ostreae*".

The project was presented at the Native Oyster Restoration Alliance (NORA) genetics working group in January 2023. NORA is the organisation that is currently most likely to adopt the use of this technique on a large scale, and the presentation was well received.

The potential of the technique was presented to the wider public at the Edinburgh Science Festival event "Oysters in Edinburgh" in April 2023.

Likewise, the project was disseminated in "The Grower" magazine. It also gained extensive mainstream and trade press coverage, including in The Times, The National, The Press and Journal, TheFishSite.com, Fish Farmer Magazine, Fish Farming Expert, Hatchery FM, Seafood Source, Insider.co.uk, Lardermag.co.uk and Global Seafood.