COMPLETED PROJECT CASE STUDY

ASSESSING THE USE OF CARDIAC BIOMARKERS FOR EARLY DIAGNOSTIC OF CARDIOMYOPATHY SYNDROME

PARTNERS

Cooke Aquaculture UK | University of Edinburgh | Life Diagnostics Ltd | Benchmark Genetics | Moredun Research Institute

PROJECT LEADS

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BACKGROUND

The incidence of cardiomyopathy syndrome (CMS) has a serious impact on Atlantic salmon (Salmo salar) production. The aetiological agent of CMS is the piscine myocarditis virus (PMCV), which primarily targets the heart. The biggest economic impact of CMS is, therefore, due to cardiac failure and sudden death of apparently healthy, pre-harvest fish. Compounding this, earlier onset of the disease has been recently noted, with reduced growth/heightened base level mortality of smaller fish. No vaccines or treatments are available against CMS.

An early indication of the onset of clinical disease would allow salmon farmers to improve the timing of their mitigation steps to further minimise health challenges and economic losses associated with CMS.

A diverse range of molecules are used in human and veterinary medicine to indicate the presence and determine stages of cardiac disease. The identification of novel biomarkers to improve diagnostics and their clinical application is currently an active area of research in veterinary medicine. By comparison, the use of biomarkers to assess heart disease in fish is non-existent.

The current diagnosis of CMS is based on clinical signs, histopathology and detection of viral RNA by RT-qPCR, requiring lethal sampling of valuable market-size fish. Neither of those tools allow predictive diagnostics or examination of the host's response to the virus at a population level, impeding a holistic understanding of disease evolution on farms and a more refined assessment of the impacts of the mitigation measures undertaken.

There are a number of cardiac biomarker candidates commonly used in human medicine that could be targeted for use in fish, which are structurally conserved among species, including salmon. Troponin is the main biomarker for the diagnosis of myocardial necrosis in acute coronary syndrome, and five isoforms have been identified in salmonids.

Troponin is a complex of three proteins that regulate muscle contraction, which will leak into circulation when damage to the muscle cell occurs.

For this reason, they can be measured in blood in the presence of muscle injury (i.e. they are "leakage" biomarkers). Very importantly, there are cardiac muscle and skeletal muscle specific isotypes of these proteins, which suggests these are tissue-specific biomarkers.

AIMS

- To identify and validate cardiac disease biomarkers for early/subclinical CMS diagnosis.
- To develop serological tests, such as ELISA and SPARCL™ (which can potentially be used on the farm) for early diagnosis of CMS, based on easy, cost-effective, non-lethal methods.

Ultimately, the findings of this project will identify CMS in the early/subclinical stages and allow a better assessment at population level, enabling farmers to identify the best mitigation strategies in sufficient time to improve farms and fish resilience to this health challenge.

IDENTIFICATION AND VALIDATION OF CARDIAC DISEASE BIOMARKERS FOR EARLY OR SUBCLINICAL CMS DIAGNOSIS

Novel cardiac biomarker candidates were identified by serum proteomic analyses of negative controls and CMS infected fish samples. Simultaneously, assays for the measurement of skeletal muscle troponin C and cardiac muscle troponin C were developed and validated using field and controlled challenge samples. Finally, the project assessed a commercially available kit for the measurement of salmon troponin in serum.

WORK DONE

A CMS sample biobank was generated, and its samples were characterised for CMS severity. The biobank created provided the samples to conduct the project and included samples from a previous study on CMS (pump-priming). The biobank built during this project has two parts: 1. field samples provided by Cooke Aquaculture, whereby a set of case definitions was drafted to classify these samples – negative control, CMS clinical and CMS subclinical case. 2. samples and genotype data provided by Benchmark Genetics from PMCV challenged smolts, which took place at VESO Aqualab in Norway.

A commercially available ELISA test kit was examined for the detection of troponins against a subset of samples from the CMS sample biobank.

Novel cardiac markers were identified from CMS-infected serum through proteomic techniques conducted in two distinct phases.

In the first phase, pre-existing pump-priming serum samples were analysed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), a qualitative proteomics tool. PCMV qPCR data were used to characterize these samples, which were arranged to allow cases vs controls study comparing fish with clinical CMS vs negative control fish. This analysis gave a list of protein sequences, allowing to group, consolidate and compare them to create a list of proteins only found in CMS+ and CMS- serum. Each protein was assessed theoretically for its biomarker significance.

In the second phase, serum samples randomly chosen from the CMS biobank were analysed using iTRAQ, a semi-quantitative proteomic tool. For this phase, both subclinical and clinical fish were included to compare against the negative controls, enabling the identification of putative biomarkers of subclinical CMS.

To improve the understanding and evaluate cardiac biomarkers gene expression in the cardiac tissue of CMS diseased fish in the field, cardiac tissue samples were randomly selected to provide an approximately equal spread of negative, low, moderate, and high cardiac PCMV load. Once RNAseq analysis was complete, the list of proteins obtained with the iTraQ proteomic analysis was matched with the list of genes overexpressed in subclinical CMS cases.

OUTCOMES

As expected, during field sampling, severe CMS cardiac lesions were more frequent in clinical CMS cases than in subclinical CMS cases. Also, clinical CMS cases had significantly higher PMCV cardiac loads than subclinical CMS cases.

Unexpectedly, there was no significant correlation between cardiac PMCV load and cardiac CMS lesion scores. This contrasts with previous results in controlled challenge studies and is suspected to be a result of the heterogeneity of infection and clinical disease dynamics in the field.

The readings obtained from the commercially available salmon cardiac troponin ELISA test kit are comparable to those obtained with the tools developed during this study. However, the test kit lacked user information consistency between different sample dilutions, a finding which is strongly suggestive of the presence of matrix effects in this assay rather than analyte effects. For these reasons, the kit assessed was not considered suitable for the purpose of clinical detection of early CMS and further testing work was not pursued.

During the qualitative proteomics study, it was established that CMS cases have detectable levels of PMCV in serum, which allowed the establishment of a case definition for CMS for that study - a CMS case is a fish that presents clinical signs and gross lesions of CMS and has detectable levels of PCMV in serum.

Interestingly, most of the proteins unique to CMS+ serum pools revealed by this study had been used as cardiac disease biomarkers or had been considered putative cardiac disease biomarkers in other species.

The semi-quantitative proteomic analysis allowed the identification of differences in serum protein profiles. Most of the proteins detected by the previous qualitative study were detected in this study too. Further, some proteins found in higher concentrations in the serum of clinical CMS cases could, in theory, be useful as prognostic biomarkers. These findings suggest that Fibulin, Lumican, and Fibrinogen are putative biomarkers of PMCV viral load in subclinical CMS cases. Unexpectedly, none of them had a “dose-response” effect on CMS cardiac lesion severity, therefore, no putative serum biomarker to estimate CMS cardiac lesion severity was identified.

Exploratory analysis of the RNAseq data using Euclidean heat maps revealed that all negative samples cluster together but that other variables cluster more loosely and there was no evident clustering by pathology scores. There was no matching between the genomic data and the proteomic data. This is not unexpected, as most of the candidate biomarkers detected by proteomics were associated either with leakage from damaged myocytes or as part of a systemic host response (i.e. acute phase proteins).

DEVELOPMENT OF COST-EFFECTIVE AND NON-LETHAL SEROLOGICAL TESTS

Serological assays for cardiac biomarkers were developed using salmon-specific antibodies. These antibodies have a higher level of sensitivity than the commercial kits developed using human or other species targets and were incorporated into two serology assays platforms for field tests.

WORK DONE

Several PCMV culture attempts were made using frozen PMCV infected cardiac tissue provided by Cooke. These culture attempts were unsuccessful, and PMCV remains non-culturable at present.

The same material was used to purify PMCV viral particles according to pre-existing viral purification protocols. These were followed by transmission electron microscopy (TEM) analysis, viral particles were present in the fluid, and the fluid was positive for PCMV by RT-qPCR, suggesting purification was successful. However, TEM analysis also revealed debris, and several attempts at removal of this were unsuccessful.

The consortium organised rabbit immunisation with the PMCV ORF1 recombinant peptide produced for this project. However, tests on polyclonal antibodies obtained did not cross react with PMCV infected heart tissue or purified PMCV. These results indicated that the serum produced in this study was not helpful in detecting PMCV. For this reason, anti-PMCV IHC development was not viable within this project. It is possible that ORF-1 is not immunogenic to rabbits, and further work would be required to assess if this is the case, but this was considered out of the scope of the project.

However, the project developed pairs of antibodies against skeletal muscle troponin C (skTnC) and cardiac muscle troponin C (cTnC) and purified these proteins for assay standardisation. These antibodies were used to produce ELISA and SPARCL assays for the specific detection of troponins in serum. SPARCL assays are based on two antibodies binding to a single antigen. These two antibodies are labelled with Acridan and horseradish peroxidase respectively, which produce a luminescence signal in the presence of an oxidising agent. This can be measured and is directly proportional to the amount of antigen in the sample. The most relevant finding and advantages are that this assay is fast (45-minute incubation), does not require repeated washes, only requires a single incubation, is portable, and is conducted in a single tube that can be measured using a portable luminometer. Life Diagnostics' luminometer was tested in this study (Vetbio-1) and compared against a 96-well plate version of the SPARCL test and an ELISA test.

These antibodies were tested in immunohistochemistry against the cardiac and skeletal tissue using biobank samples. The immunohistochemical pattern indicates that the cTnC assay will detect damage in the myocardium and slow twitch skeletal muscle, whereas the skTnC assay will detect damage in the fast twitch muscle.

All samples of the field biobank and controlled challenge were tested with cTnC and skTnC assays. cTnC serum concentration variations were assessed according to PCMV cardiac load and CMS cardiac lesion severity. cTnC and skTnC concentrations in serum were tested for clinical cases of Pancreas Disease (PD) as well.

OUTCOMES

The anti-PCMV ELISA prototype assay was tested with field serum samples from the biobank. The readings from this assay were inconsistent across replicates, suggesting further refinement would be necessary to develop a robust test. This failure is suspected to have resulted from contamination of the purificate with remnants of cardiac tissue. As a result of the above, serological assessment of PMCV exposure in CMS infected fish was not possible.

Troponin SPARCL assays were also tested with field serum samples from the biobank. cTnC serum concentrations were significantly higher in CMS subclinical (and clinical) cases than in negative controls, suggesting that cTnC is a good candidate for the detection of subclinical CMS. Preliminary diagnostic parameters for the assay's detection of subclinical CMS were calculated. For example, the results suggest the assay would require a random sample of serum from 20 fish to detect a CMS prevalence of 70% in the population, whereas a sample of 30 fish would be able to detect a prevalence of slightly over 50%. Interestingly, there was no significant correlation between serum cTnC concentrations and PMCV viral load, and there was no increase in cTnC concentrations correlated with CMS cardiac lesion severity. These findings suggest that although cTnC is a biomarker of subclinical/clinical CMS, it is not a biomarker of CMS severity or viral load in CMS diseased fish.

CMS challenged fish showed no significant differences in cTnC or skTnC serum concentrations due to CMS cardiac lesion severity or PCMV cardiac load. A similar result was also noted when stratifying the data by genotype. Overall, these results are consistent with those noted in the field, where neither cTnC nor skTnC concentrations were biomarkers of CMS lesion severity or PCMV cardiac load.

cTnC and skTnC levels are significantly increased in serum of PD fish too with no overlap with controls. In the presence of skeletal muscle damage in mid-late PD, the increase in the serum concentration of cTnC and skTnC is an order of magnitude higher than that noted for CMS. This indicates that these differences in magnitude can be used to differentiate between CMS and PD when using cTnC as a biomarker in the field.

Overall, these results are extremely positive and suggest that the cTnC assay developed, as well as any assay based on cTnC detection, will be useful in the management of subclinical CMS in the field. The diagnostic power may be improved by its inclusion in a panel of serological tests and further technical development of the assay, as well as knowledge of its field temporal dynamics to inform the interpretation of the test.

IMPACT

The Atlantic salmon aquaculture industry is a major contributor to the Scottish and UK economy. Cardiomyopathy syndrome (CMS), caused by piscine myocarditis virus (PMCV), has had a major impact on this production, with reported CMS outbreaks increasing dramatically in recent years. Monthly mortality reports compiled by the Scottish Salmon Producers Organisation [now Salmon Scotland] show CMS as one of the leading health issues affecting the Scottish industry. The most significant economic impact of CMS is the loss of apparently healthy fish, dying suddenly of heart failure just before harvest. Early diagnosis of CMS is paramount to enable salmon producers to take steps to avoid losses during this important stage in the production cycle.

The outputs of this research have made a major contribution to the development of an integrated toolkit for Atlantic salmon cardiac health. It has generated a toolkit that will be adaptable to other cardiac diseases, such as pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI).

Life Diagnostic Ltd can expand into the aquaculture market, with a CMS diagnostic tool that can be applied in a portable platform (VetBio-1), which is a major advantage for aquaculture health professionals working in remote locations.

Benchmark Genetics could further develop and improve the evaluation of CMS resistance stocks.

These results are the basis for the follow-on SAIC-funded project – Use of serum biomarkers for early differential diagnostics of cardiomyopathies of Atlantic salmon: field and challenge assessment – ensuring that data, techniques, and expertise will continue to be shared, thus extending beyond the lifespan of the project the academic and commercial benefits of the research undertaken.

ADDITIONAL INFORMATION

Knowledge exchange has taken place through different avenues throughout the duration of the project. This has involved primarily the project consortium, and then wider scientific community, Scottish aquaculture industry (producers, breeders, feed manufacturers, pharmaceutical companies and retailers), as well as government policy makers.

Presentations

Two presentations at PD Trination, 22 April 2021:

- 'Identification of serum proteins from Atlantic salmon with CMS' by Janina Costa, MRI
- 'Assessing the use of cardiac biomarkers as a health management tool for early diagnostic of CMS in Atlantic salmon' by Jorge del Pozo

Articles

Janina Z. Costa, Jorge del Pozo, Kevin McLean, Neil Inglis, Philippe Sourd, Andrei Bordeianu, Kim D. Thompson (2021) Proteomic characterization of serum proteins from Atlantic salmon (Salmo salar L.) from an outbreak with cardiomyopathy syndrome. Journal of Fish Diseases, DOI:10.1111/jfd.13488

Three more articles are planned, including a description of the characterisation of troponin assays in the field sample, semiquantitative proteomic candidate biomarkers, and CMS field genomic data analysis.

NEWS ARTICLES

Diagnostic tool designed to improve fish heart health - FishFarmingExpert.com

CMS is a growing problem in salmon farming - FishFarmingExpert.com

Researchers plan simpler, faster salmon heart disease test - FishFarmingExpert.com

Researchers promise non-lethal mass testing system for cardiomyopathies (salmonbusiness.com)

Early warning system aims to get to the heart of the salmon disease, CMS (salmonbusiness.com)

Developing a mass testing tool for fish heart diseases | The Fish Site

Researchers look to new ways to tackle CMS in salmon | The Fish Site

Researchers develop 45-minute salmon heart health test - Undercurrent News

Scottish researchers look for early CMS detection in salmon - Hatchery International

Scottish researchers look for early CMS detection in salmon - Aquaculture North America

Early warning system aims to get to the heart of salmon disease (fishfocus.co.uk)

Early warning system aims to get to the heart of salmon disease (sustainableaquaculture.com)

Researchers look to new ways to tackle CMS in salmon (2lua.vn)

Aquaculture experts and scientists team up to prevent killer disease in salmon - Business Insider

Fast test to detect deadly heart disease – Fish Farmer Magazine