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Report 10 · 2014

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Publisher
Norwegian Veterinary Institute · PO Box 8156 Dep. · N-0033 Oslo · Norway

Cover design: Graf AS
Photo frontpage: Biopix

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ISSN 1890-3290 electronic edition

Suggested citation:
Vågnes, Ø., Gulla, S, Colquhoun, D., Biering, E. Development of injection an cohabitation challenge models for *Vibrio anguilarum* serotype O1 in farmed ballan wrasse *(Labrus bergylta)*. Veterinærinstituttets rapportserie 10-2014. Norwegian Veterinary Institute; 2014.

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Ordered by

The Norwegian Seafood Research Fund - FHF

11.06.2014

ISSN 1890-3290 electronic edition

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Summary

The FHF funded project 900818 – *Cleaner fish: Causes of loss and preventive measures* aims to facilitate development of vaccines for cleaner fish by developing infection (challenge) models for some important bacterial pathogens. This report describes 4 trials involving intraperitoneal, bath and cohabitant based exposure in ballan wrasse (*Labrus bergylta*). Two strains of *Vibrio anguillarum* serotype O1, one originally isolated from farmed Atlantic salmon and one from captive, wild-caught ballan wrasse were utilised. Only one strain (the Atlantic salmon isolate) induced mortality levels suitable for vaccine testing when given by intraperitoneal (i.p.) injection. Bath or cohabitation exposure did not lead to satisfactory levels of mortality for use in vaccine efficacy testing. *Vibrio anguillarum* was re-isolated from all (exposed) dead fish examined. We conclude that the intraperitoneal model is promising for testing of vaccines against *Vibrio anguillarum* in ballan wrasse, although pre-challenges will be necessary to establish virulence levels in different combinations of bacterial strain and fish stocks.

Summary in Norwegian

Det FHF finansiert prosjekt *900818 – Rensefisk: Tapsårsaker og forbyggende tiltak* har som delmål å bidra til utvikling av rensefiskvaksiner ved å utvikle smittemodeller for noen viktige sykdomsfremkallende bakterier. Denne rapporten beskriver fire studier der berggylt (*Labrus bergylta*) ble smittet intraperitonealt, ved badsmitte og som kohabitanter. To stammer av *Vibrio anguillarum* serotype O1, en isolert fra oppdrettslaks og en fra villfanget berggylt ble testet. Bare laksestammen induserte dødelighet på et nivå som er egnet for vaksineuttesting når de ble gitt ved intraperitoneal (i.p.) injeksjon. Bad og kohabitant eksponering resultert i ingen eller lav dødelighet og derfor å betrakte som uegnet for bruk i vaksinetesting. *Vibrio anguillarum* ble reisolert fra alle (eksponert) døde fisk undersøkt. Vi konkluderer med at den intraperitoneale (i.p.) injeksjonsmodellen er lovende for testing av vaksiner mot *Vibrio anguillarum* i berggylt, men pre-smitte blir nødvendig for å etablere virulensforhold mellom den utvalgt bakterien og den aktuelle fiskepopulasjon.

Contents

Introduction

Effective vaccines against the most important bacterial diseases have been crucial for the advance of salmonid aquaculture, and bacterial infections will probably continue to hamper the production and use of cleaner fish until commercial vaccines against the most common diseases become available. The FHF funded project *900818 – Cleaner fish: Causes of loss and preventive measures* aims to facilitate development of vaccines for cleaner fish by developing infection (challenge) models for some important bacterial pathogens. This report deals with ballan wrasse (*Labrus bergylta*) and the bacterium *Vibrio anguillarum* serotype O1.

Materials and methods

Experimental fish and holding conditions

Ballan wrasse were obtained from a commercial cultivation site located in Hordaland county, western Norway. The fish were first generation offspring of locally wild-caught broodstock. The experimental fish (approximately 20 – 50g) were transported to the Institute of Marine Research (IMR) at least one week prior to experimental infection. The water temperature was gradually increased to 15°C. Before each handling, 10 fish at a time were anesthetized (benzocaine 0.6 g/L, metomidate 0.05 g/L). The fish were starved from two days before infection until one day after infection. Where marking of different groups of fish was necessary, fish were marked in the right cheek with visible implant elastomer (VIE).

Bacterial strains

Two strains of *V. anguillarum* serotype O1 were utilized. Strain F48 (Bergen) was isolated from a wild caught ballan wrasse used as cleaner fish in a commercial aquaculture site for Atlantic salmon on the west coast of Norway, while strain F47 (Bergen) was isolated from farmed Atlantic salmon displaying macroscopic and histological changes consistent with vibriosis. The strains were phenotypically characterized, serotyped by slide agglutination using antisera raised against a range of *V. anguillarum* serotypes. The isolates were stored at -80°C in appropriate liquid media containing 20 % glycerol.

Prior to enrichment for infectious challenge the bacteria were grown on blood agar (5 % sheep blood + 2 % NaCl). Cultures for infectious challenge were produced by inoculation of 500ml marine broth with 0.5ml of a 24 hour pre-culture in the same medium, which was harvested after approximately 18 hrs (late exponential phase). The bacterial density of infection cultures was established by serial dilution and colony counting.

Sampling

The experimental fish were observed daily and bacteriology (blood agar with 2% NaCl, BAS) was performed on all mortalities. Formalin fixed samples were taken from selected fish (detailed below). Control samples (bacteriology and formalin fixed tissues) were taken from 5 fish prior to initiation of each challenge trial. No relevant signs of disease were observed or bacteria detected in the experimental '0' samples.

Challenge trials

Pilot trial intraperitoneal (i.p.) injection

40 fish (20 – 50g) were distributed into groups of 10 fish in 4 separate tanks holding 250 liters water at 15 °C. Each group was then injected with either one of three different doses (10^5, 10^6 and 10^7 cfu suspended in 0.1 ml saline) of the ballan wrasse isolate or the Atlantic salmon isolate (10^7 cfu). The fish were observed for 12 days, with daily mortality removal. All dead fish were subjected to bacteriology investigation involving plating of kidney tissues on BAS followed by incubation at 15°C. Formalin fixed tissues were sampled from those fish considered to be non-cadaverous. The identities of single isolates of each strain were confirmed by the Norwegian Veterinary Institute Bergen, otherwise visual observation of colony morphology typical for *V. anguillarum* was used to confirm bacterial identification.

Main i.p. injection trial

370 ballan wrasse (20-50g) were used. The experimental fish were randomly distributed in 8 tanks of 250 litres (46-47 fish per tank, water temperature 15 ºC, flow 450 litres/h).

Fish in three tanks (1, 2 and 6) were i.p. injected with 0.1 ml (10^7 cfu) of wrasse strain F48 while three tanks (3, 5 and 7) were injected with (10^7 cfu) of salmon strain F47. Fish in two control tanks (4 and 8) were injected with 0.1ml 0.9% NaCl. Serial sampling for bacteriology and formalin fixed tissues were taken from tank 3 (salmon strain) and tank 6 (wrasse strain). The fish were observed for 18 days, when the remaining surviving fish were euthanised and sampled for bacteriology.

Bacterial samples were collected during the observation period from all dead wrasse in tanks 1, 2, 5 and 7. Bacterial samples were also taken from all surviving fish in these tanks at the end of the study as well as 20 control fish. Bacterial samples were taken from renal tissue and cultivated on blood agar added 2 % NaCl at 15 °C.

Pilot Bath infection model

A pilot bath–infection trial was conducted in which 4 groups of 10 fish were exposed either to the salmon isolate or the wrasse isolate at 10^5 and 10^7 cfu ml^{-1} for 30 minutes in a water volume of 50 liters at 15°C. The fish were observed daily for 19 days and dead fish removed daily and examined bacteriologically. Surviving fish were also examined bacteriologically at the end of the trial.

Main bath infection model

A bath–infection trial was conducted in which 4 groups of 40 fish were exposed either to the salmon isolate or the wrasse isolate at 10^7 cfu ml^{-1} (i.e duplicate treatments) for 30 minutes in a water volume of 100 liters at 15°C. The fish were observed daily for 19 days and dead fish removed daily and examined bacteriologically. Surviving fish were also examined bacteriologically at the end of the trial.

Intraperitoneal/cohabitation infection model

8 groups of ballan wrasse were split into 8 tanks each containing 66 fish (20-50g). In three tanks 33 fish were injected intraperitoneally with 10^7 *V. anguillarum* F48 (from wrasse) and in three tanks 33 fish were injected intraperitoneally with 10^7 *V. anguillarum* F47 (from salmon). In two tanks 33 fish were injected intraperitoneally with 0.1 ml saline. The Injected fish in each tank were marked using VIE. The experiment ran for 21 days with daily removal of mortalities which were subjected to bacteriological examination. At termination 10 surviving injected and cohabitant fish (for each bacterial strain) were euthanized and subjected to bacteriological examination.

Results

Pilot intraperitoneal trial

The accumulated mortality for each dose/isolate is illustrated in Figure 1.
A direct dose response with the wrasse isolate F48 in wrasse was not achieved. Generally the wrasse isolate appears to be less pathogenic for wrasse than the salmon isolate (F47) at a similar level of challenge.

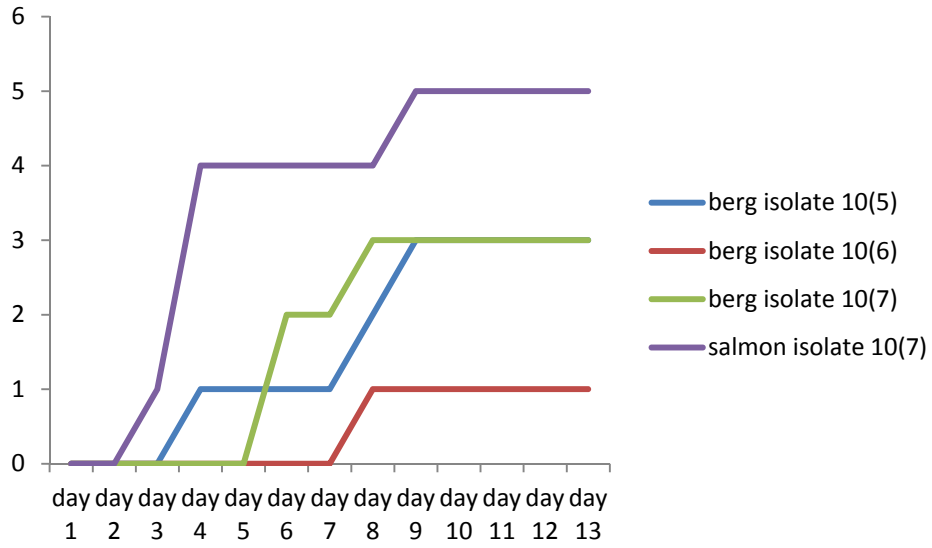

Figure 1: accumulated mortality (number of fish) in the intraperitoneal pre-trial. (berg isolate = F48 isolated from ballan wrasse; salmon isolate = F47 isolated from Atlantic salmon.

Main intraperitoneal trial

No mortality was observed during initial handling or in the control fish during the study. Cumulative mortality is visualized in Figure 2. Mortality started 2 days post injection in fish injected with the salmon strain while mortality caused by the wrasse strain began on day 5 post injection. The results in this trial confirmed the earlier indication of higher virulence of the salmon strain (F47), with more acute and higher total mortality levels (40+ - 60%) than the wrasse strain F48 (10 – 20 %). Mortality had ceased in all groups when the experiment was terminated. The mortality in the sampling tanks was very similar to that identified in the tanks not disturbed by sampling.

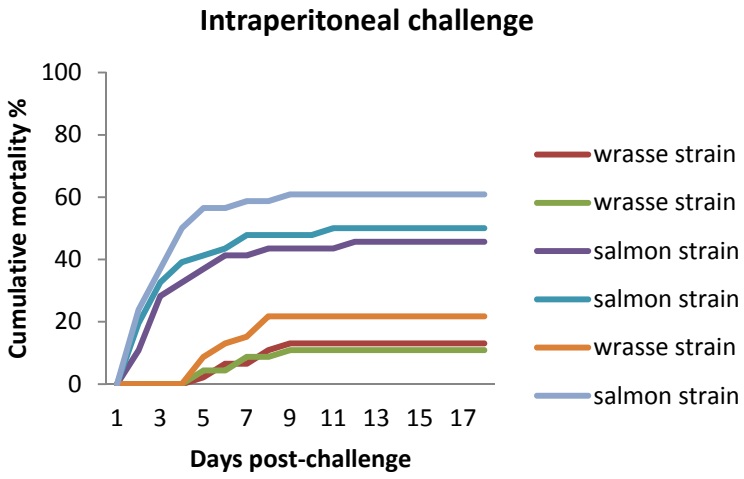

Figure 2: Cumulative mortality (%) following intraperitoneal injection of farmed ballan wrasse with V. anguillarum serotype O1 isolated from ballan wrasse and salmon.

V. anguillarum was isolated from all dead fish examined. All samples from control fish and surviving fish at the termination of the trial were negative for *V. anguillarum*.

Pilot bath exposure trial

Only one fish exposed to 10^7 cfu ml^{-1} F47 died 6 days post infection.

Main bath exposure trial

Bath exposure with wrasse isolate F48 did not lead to registered mortality during the 19 day trial. Bath exposure to the Atlantic salmon isolate F47 led only to low level mortality (Fig 3). Thus this experiment also supports the previous evidence that isolate F47 is more virulent for wrasse than strain F48 originally isolated from wrasse. *V. anguillarum* was cultured from all fish which died during the course of the experiment. Five surviving fish investigated bacteriologically did not result in growth of *V. anguillarum*.

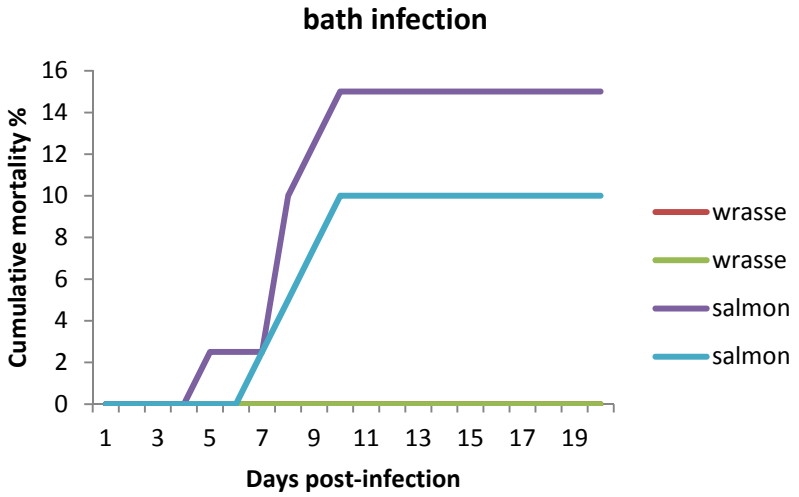

Figure 3: Mortality observed following bath exposure (10^7 cfu ml^{-1} for 30 minutes) of ballan wrasse to Vibrio anguillarum. (wrasse = F48 isolated from ballan wrasse; salmon = F47 isolated from Atlantic salmon).

Intraperitoneal/ Cohabitation trial

No mortality was observed in cohabitant fish during the observation period and *V. anguillarum* could not be cultured from surviving cohabitant fish at termination of the trial. Mortality amongst groups injected intraperitoneally followed a similar pattern to that observed in the previous intraperitoneal challenges i.e. The salmon isolate appeared significantly more virulent than the wrasse isolate. The only obvious difference (Fig. 4) between the main intraperitoneal challenge and those injected in the intraperitoneal/cohabitant trial was a slightly later onset of initial mortality caused by the salmon isolate, on day 4 compared to day 2-3 in the previous trials.

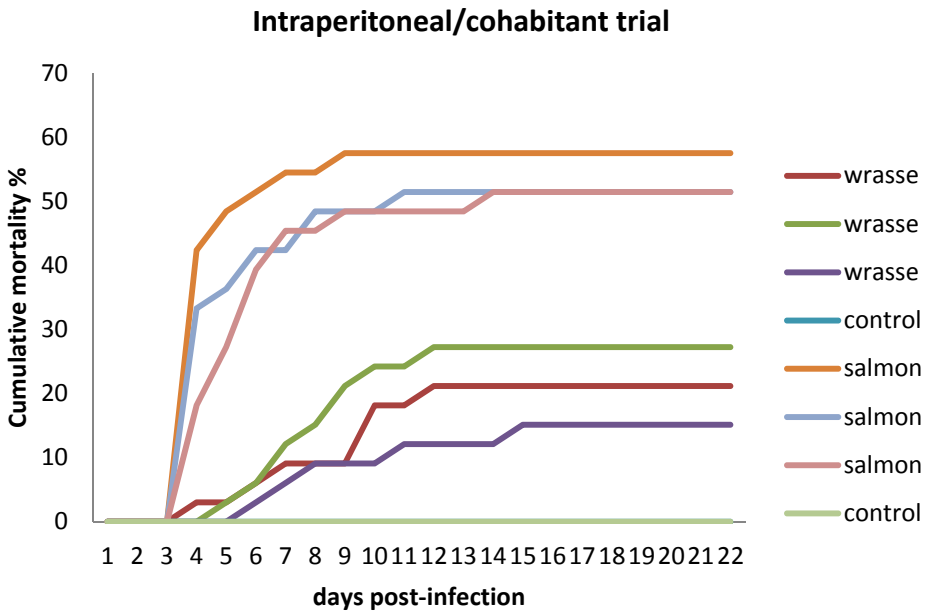

Figure 4: Mortality amongst intraperitoneally injected groups. Mortality amongst cohabitant fish was not observed (wrasse = F48 isolated from ballan wrasse; salmon = F47 isolated from Atlantic salmon).

Conclusions

The series of experiments carried out under this work package successfully fulfilled Kochs' postulates and thus confirmed *Vibrio anguillarum* serotype O1 as a 'primary' pathogen of farmed ballan wrasse. Perhaps surprisingly this work also identified considerably higher virulence for strain F47 originally isolated from farmed Atlantic salmon, than for strain F48, originally isolated from ballan wrasse. If high virulence is a trait considered desirable in a vaccine candidate strain, the results may indicate that more *V. anguillarum* strains should be screened for virulence prior to selection of a vaccine strain. Intraperitoneal injection appears to be the most robust method for both vaccine testing and virulence screening for *V. anguillarum* in ballan wrasse, as reproducible mortality levels approaching the level suitable for vaccine testing (60-70%) were only achieved on intraperitoneal injection (of salmon strain F47).

The Norwegian Veterinary Institute (NVI) is a nation-wide research institute in the fields of animal health, fish health, and food safety. The primary mission of the NVI is to give research-based independent advisory support to ministries and governing authorities. Preparedness, diagnostics, surveillance, reference functions, risk assessments, and advisory and educational functions are the most important areas of operation.

The Norwegian Veterinary Institute has its main laboratory in Oslo, with regional laboratories in Sandnes, Bergen, Trondheim, Harstad og Tromsø, with about 330 employees in total.

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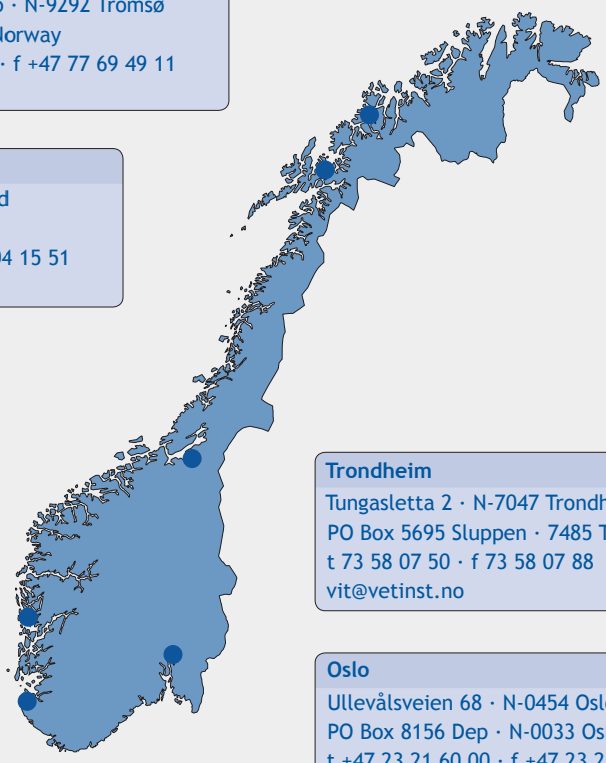

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