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Development of injection and cohabitation challenge models for atypical *Aeromonas salmonicida* in farmed ballan wrasse (*Labrus bergylta*)

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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 2 trials where ballan wrasse (*Labrus bergylta*) were infected by 2 strains of the bacterium atypical *Aeromonas salmonicida*. Both strains induced mortality levels suitable for vaccine testing when given by intra peritoneal (i.p.) injection, but only one strain induced this level of mortality by cohabitation. Atypical *A. salmonicida* was re-isolated from dead fish and found in tissue lesions by immunohistochemistry. We conclude that the described models are promising for testing of vaccines against atypical *A. salmonicida* in ballan wrasse.

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 to studier der berggylt (*Labrus bergylta*) ble smittet med to stammer av bakterien atypisk *Aeromonas salmonicida*. Begge stammene induserte dødelighet på et nivå som er egnet for vaksineuttesting når de ble gitt ved intraperitoneal (i.p.) injeksjon, men bare en av stammene indusert høy nok dødelighet når smitten ble overført ved kohabitering. Atypisk *A. salmonicida* ble reisolert fra død fisk og funnet i vevslesjoner ved immunhistokjemi. Vi konkluderer med at de beskrevne modellene er lovende for testing av vaksiner mot atypisk *A. salmonicida* i berggylt.

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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 vaccines against the most common diseases are developed and put on the market. 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 atypical *Aeromonas salmonicida*.

Materials and methods

Fish and stocking conditions

Ballan wrasse were obtained from a commercial cultivation site located in Hordaland county, western Norway. The fish were of local stock as brood fish originally had been caught adjacent to the cultivation site. The fish were transferred from the farm to tanks at the Institute of Marine Research one week before infection for acclimatization. The 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 not fed from two days before infection until one day after infection.

Bacterial strains

Two strains of atypical *Aeromonas salmonicida* designated F272-2 and F272-5 were used. The strains were isolated from two wild caught ballan wrasses used as cleaner fish in a commercial aquaculture site for Atlantic salmon on the west coast of Norway. The strains were characterized and then stored at -80°C in appropriate liquid media containing 20 % glycerol. The two strains were A-layer positive but with different morphological appearance.

Bacteria were grown on blood agar (5 % sheep blood + 2 % NaCl) at 15 °C for 5 days. Colonies from each strain were then re-suspended in saline (0.9 % NaCl) until 50 % transmittance at 520 nm. To ensure pathogenicity, the strains were passed through fish before the experiment. 6 wrasses for each strain were injected intraperitoneally (i.p) with 0.1 ml of the resuspended bacteria. Bacteria were then re-isolated from renal tissue, passaged twice on blood agar with 2 % NaCl at 15°C and frozen until used for infection.

Infection by intraperitoneal (i.p.) injection, first trial

320 ballan wrasses (average weight 50 g) were kept at 15 °C in 8 tanks of 250 liters (40 individuals in each tank). The wrasses were introduced into the tanks one week prior to infection for acclimatization. Infection and sampling were performed according to Table 1.

The wrasses were infected by i.p. injection (25 G needle) of 10^7 bacteria in 0.1 ml 0.9 % NaCl. Tissue samples were collected in 4 % formaldehyde for histopathological studies and immunohistochemistry. Bacteriological samples from renal tissue were cultivated on blood agar and blood agar added 2 % NaCl (incubated at 22 °C and 15 °C respectively). Tank 5, 7 and 8 were used for sampling. The other tanks were used for mortality registration.

Table 1: Overview of groups and sampling for injection infection of ballan wrasse with 2 strains of atypical Aeromonas salmonicida

Infection strain	Registration	Sampling for bacteriology and histopathology	Tank
F272-2	Mortality	Only dead fish	**1**
F272-2	Mortality	Only dead fish	**3**
F272-2	Histopathology /bacteriology	Dying fish + 5 individuals/14 days	**7**
F272-5	Mortality	Only dead fish	**2**
F272-5	Mortality	Only dead fish	**6**
F272-5	Histopathology /bacteriology	Dying fish + 5 individuals/14 days	**5**
Saline - control	Control mortality	Only dead fish	**4**
Saline - control	Histopathology /bacteriology	5 individuals /14 days	**8**

Infection by i.p. injection and cohabitation, second trial

560 ballan wrasses with average weight 48 g were used. The wrasses were kept in 8 tanks of 250 litres (70 fish in each tank, water temperature 15 ºC, flow 450 litres/h). The fish were introduced into the tanks one week prior to infection for acclimatization. In each infection-tank, 35 fish were i.p. injected as described above, and 35 uninfected individuals were added as cohabitants. The infection and sampling setup was as described in Table 2 below.

All i.p. infected wrasses were labelled at the base of the anal fin with yellow VIE marking (North West Marine Technology Inc.). To enhance infection pressure from the injected fish, removal of the first dead fishes was delayed by 24 hours.

Bacterial samples were collected from all dead wrasses in tanks 2 and 5. At the end of the study bacterial samples were collected from all remaining wrasses in tanks 2 and 5 and from 20 control fish. Bacterial samples were taken from renal tissue and cultivated on blood agar at 22 °C and on blood agar added 2 % NaCl at 15 °C.

Table 2: Overview of groups and sampling for cohabitation infection of ballan wrasse with 2 strains of atypical Aeromonas salmonicida.

Infection strain	**Registration**	**Sampling for bacteriology and histopathology**	**Tank**
F272-2	Mortality		**1**
F272-2	Mortality	Dead fish (bacteriology) and survivors (histology and bacteriology)	**2**
F272-2	Mortality		**3**
F272-5	Mortality	Dead fish (bacteriology) and survivors (histology and bacteriology)	**5**
F272-5	Mortality		**6**
F272-5	Mortality		**7**
Saline - control	Control mortality		**4**
Saline - control	Control mortality	Survivors	**8**

Production of polyclonal rabbit antisera against atypical *Aeromonas salmonicida*

Bacteria from each strain were cultivated on blood agar for 4 days at 22 °C. Colonies were then harvested and inactivated overnight at 4°C with 0,26 % formaldehyde in phosphate buffered saline (PBS). The bacteria were then washed in PBS three times with centrifugation at 400 x g for 10 minutes and thereafter resuspended in PBS to an approximate concentration of McFarland 3-4. The inactivated and washed bacteria were then shipped to Eurogentec for production of polyclonal antisera in rabbits.

Histopathology and immunohistochemistry

Tissue was fixed in 4 % formaldehyde, embedded in paraffin and sectioned at 2 µm before mounting on slides and stained with haematoxylin-eosin. The samples were then examined by light-microscopy. Immunohistochemistry was performed on slides with standard methods. The antisera against atypical *Aeromonas salmonicida* were diluted 1:5000.

Results

I.p. infection model

No mortality was observed due to initial handling or in the control fish during the study. Cumulative mortality is visualized in Figure 1. It started 4 days after infection in all tanks infected with strain F272-2. Mortality in fish infected with strain F272-5 was first observed at Day 6. In general, the disease progression was more acute for strain F272-2 than for strain F272-5. Total cumulative mortalities in tanks with fish infected with F272-2 were 90 % in tank A1 and 80 % in tank A3 (Fig 1). Total cumulative mortalities in tanks with fish infected with strain F272-5 were 80 % in tank A2 and 70 % in tank A6 (Fig1). Mortality had ceased in all groups when the experiment was terminated. The mortality in the sampling tanks were on par with this, but those values are not included in Fig.1 as mortality progression will be influenced by sampling of live fish during the experiment.

Atypical *A. salmonicida* was isolated from all dead or moribund fish infected with F272-5, and from all but 2 dead or moribund fish infected with F272-2. Samples from these two individuals were overgrown with intestinal bacteria. All samples from control fish were negative for atypical *A. salmonicida*. At the end of the experiment, 18 renal samples were collected from wrasses infected with strain F272-2 and 37 samples were collected from fish infected with strain F272-5. Only 2 of the F272-2 and 3 of the F272-5 infected samples were positive for atypical *A. salmonicida*.

Histological examination revealed no changes in the control fish. In infected fish, bacterial micro colonies were observed. These were found to be positive by immunohistochemistry using antisera made against atypical *A. salmonicida* (Fig 2). We did not find bacterial micro colonies in tissue of surviving fish. Instead we observe granulomas with necrotic interior.

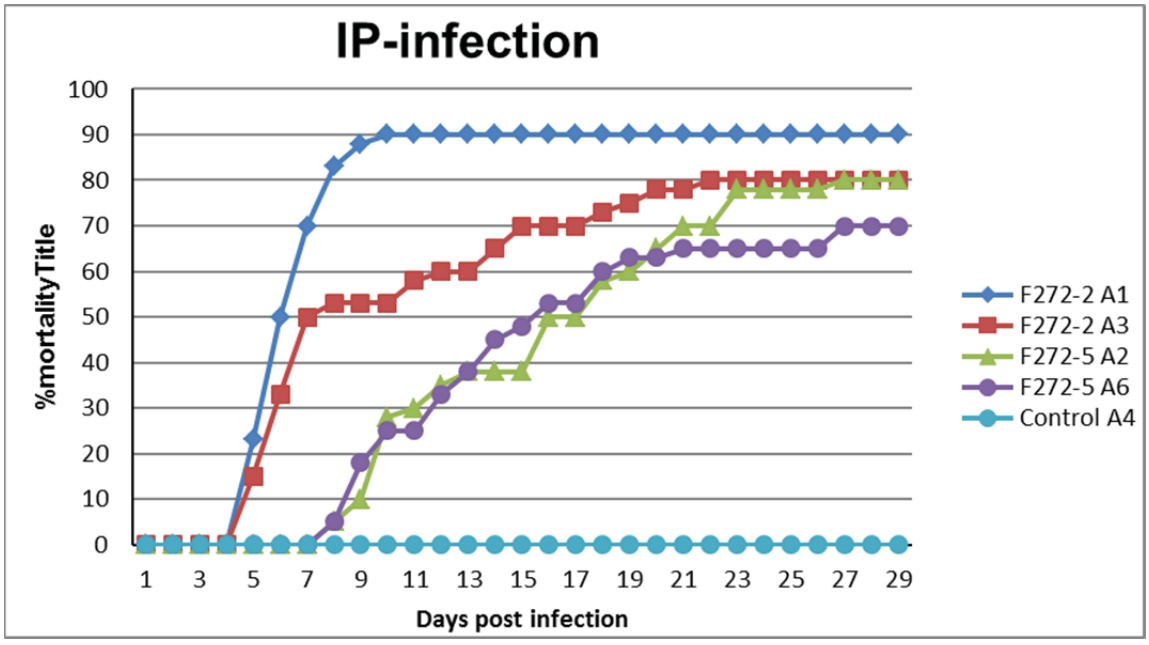

Figure 1: Mortality rate of i.p. infected and control ballan wrasse. Tanks A1 and A3 are infected with atypical A. salmonicida strain F272-2. Tanks A2 and A6 are infected with atypical A. salmonicida strain F272-5.

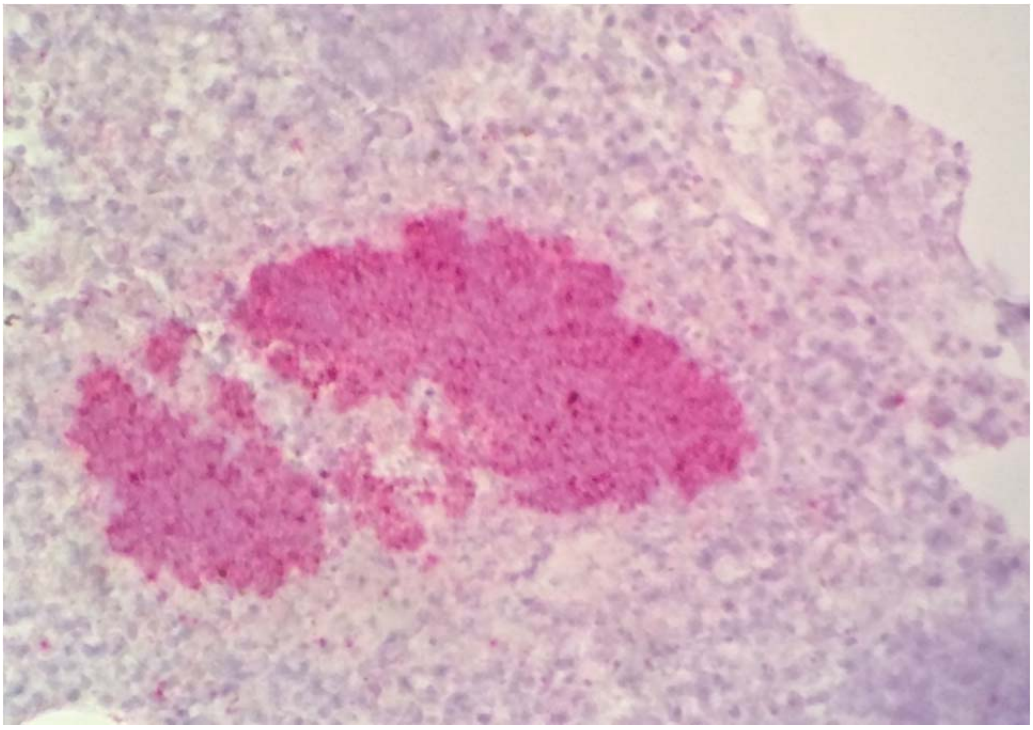

Figure 2: Micro colony in spleen of ballan wrasse i.p. challenged with atypical A. salmonicida strain F272-5. Immunohistochemistry with F272-5 specific antisera.

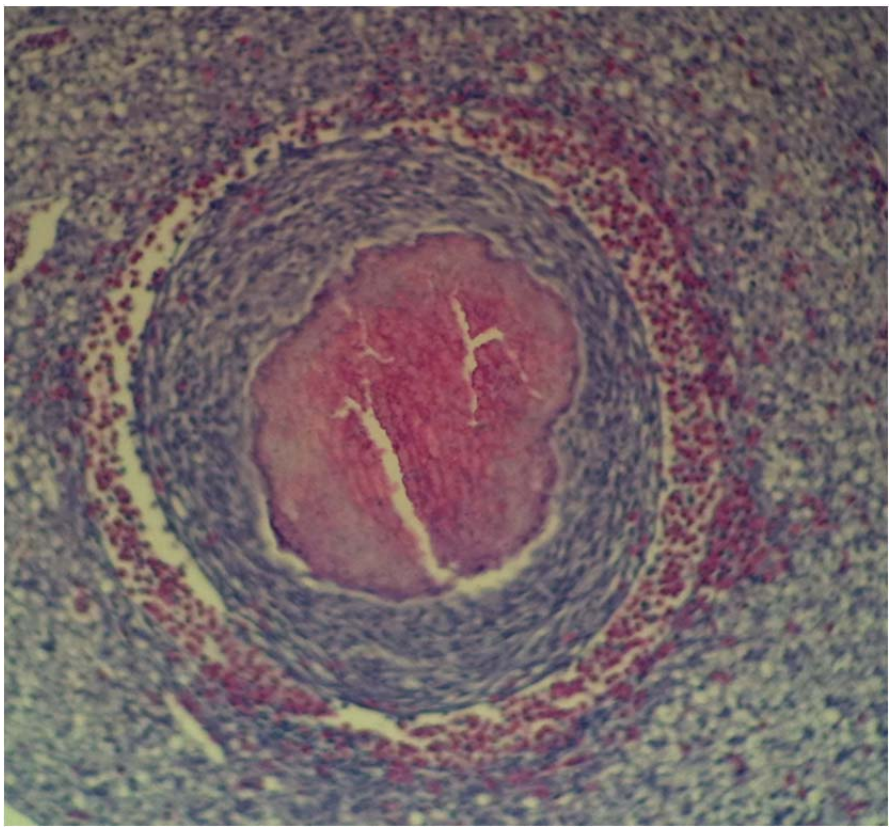

Figure 3: Granuloma from end sampling in the intraperitoneal infection trial with necrotic acellular contents.

Cohabitation infection model

No mortality was observed in the control fish. The first mortalities occurred 4 days after infection among F272-2 infected fish in tanks 1 and 3. The F272-5 infected fish in tanks 5, 6 and 7 started to die 5 days after infection. Mortality rates were similar for all tanks until two weeks after infection. At this time mortality ceased in the F272-2 infected fish but continued to increase among the F272-5 infected fish. Cumulative mortalities in total and following either i.p. injection or cohabitation infection are shown in Table 3.

7 days after infection, the first cohabitant infected with F272-5 died in tank 5. However, we were not able to re-isolate atypical *A. salmonicida* from this fish. The first dead cohabitants positive for atypical *A. salmonicida* died in tank 3 (F272-2) 14 days after infection. Mortality progression due to i.p. infection is shown in Fig. 4 and due to cohabitation infection in Fig. 5.

Atypical *A. salmonicida* were re-isolated from all but 3 dead fish infected with F272-2 and all but 5 dead fish infected with F272-5. Similar to the first study with i.p. infection, histological examinations revealed micro colonies that stained positive for atypical *A. salmonicida*. Such colonies were found in wrasse infected by both routes and with both isolates. When survivors were tested at termination of the study, 0 of 20 F272-2 infected fish and 2 of 20 F272-5 infected fish tested positive for atypical *A. salmonicida*.

Table 3: Cumulative mortalities in the cohabitation study.

Bacterial strain	Total (%)	I.p. injection (%)	Cohabitation (%)
F272-2, tank 1	40	74	6
F272-2, tank 2	37	66	9
F272-2, tank 3	40	71	9
F272-5, tank 5	61	89	34
F272-5, tank 6	74	89	60
F272-5, tank 7	74	89	60

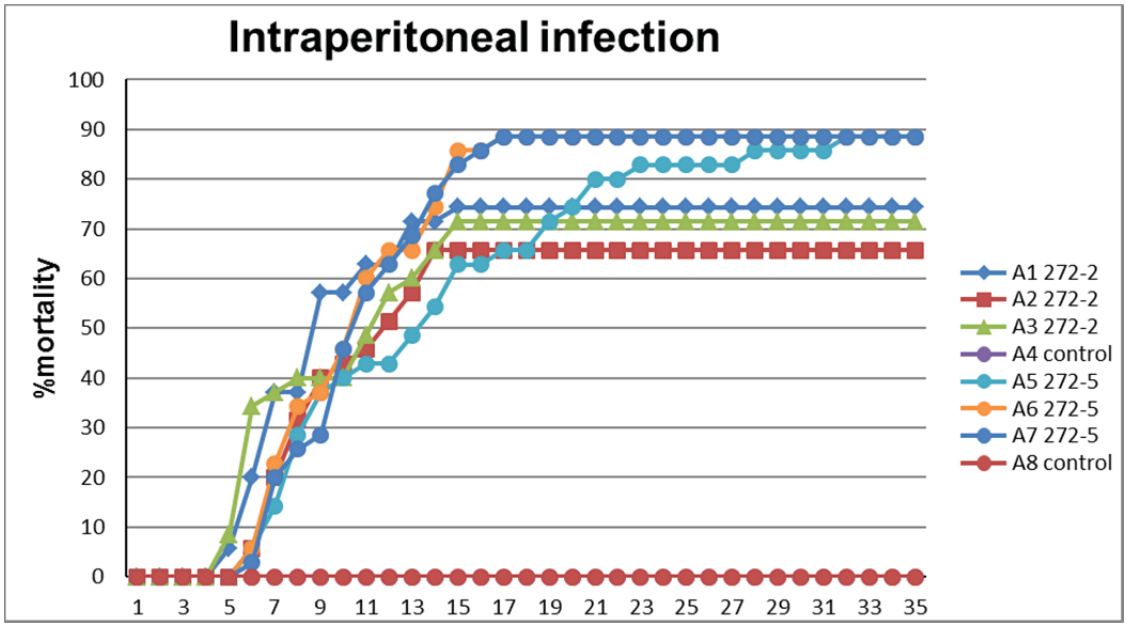

Figure 4: Mortality rates for i.p. infected ballan wrasse.

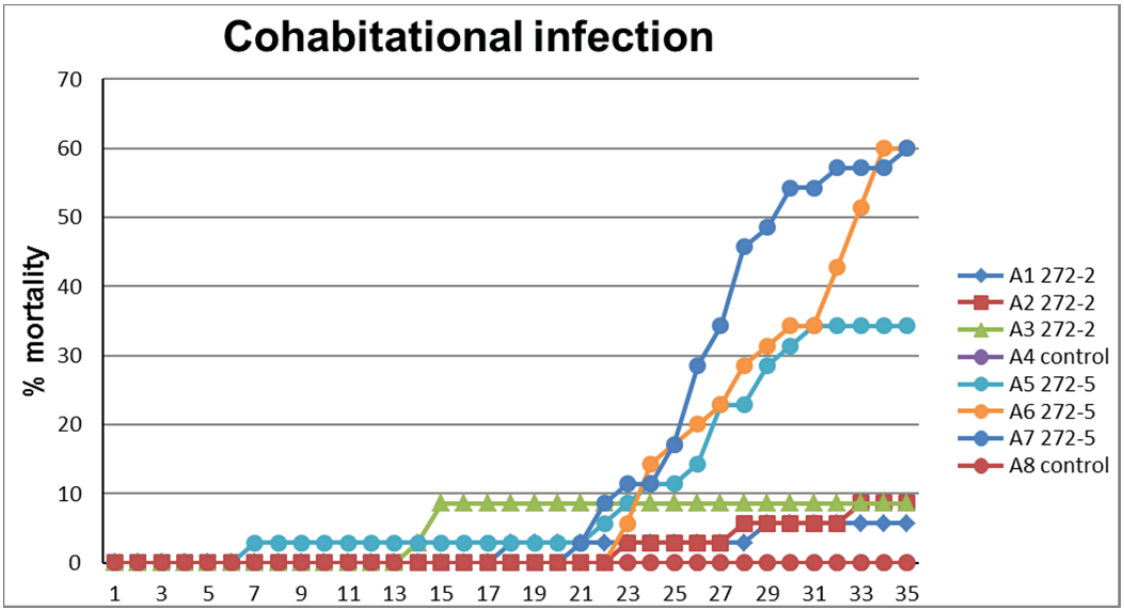

Figure 5: Mortality rate for ballan wrasse infected by cohabitation.

Conclusions

The first i.p infection with strains F272-2 and F272-5 showed that farmed ballan wrasse i.p. injected with 10^7 atypical *A. salmonicida* will develop disease and mortality. In this case, the mortality ranged between 80 - 90 % for fish infected with strain F272-2, and 70 - 80 % for fish infected with strain F272-5. In the second experiment, strain F272-5 induced the highest mortality following i.p. injection with 89 % in all tanks, compared to 66 - 74 % for strain F272-2. All levels of cumulative mortality following i.p. infection observed in these studies are within acceptable limits for vaccine testing. In general, low mortality in infection tests makes it difficult to distinguish vaccines with low efficacy from vaccines with high efficacy. On the other hand, 100 % mortality makes it impossible to accurately calculate protection offered by the vaccine and to compare groups.

Atypical *A. salmonicida* was re-isolated from nearly all diseased fish, and the bacteria were found in tissue lesions. This confirms that atypical *A. salmonicida* caused the mortality observed during the trials. However, survivors were mostly negative for atypical *A. salmonicida*. This shows that some individuals are able to neutralize the bacterium, even when it is given by i.p. injection.

Cohabitation is a more natural route of infection compared to injection. Furthermore, during i.p. infection of vaccinated fish, bacteria are usually injected at the same location as the fish was previously vaccinated. Vaccination with oil-adjuvanted vaccines will induce potent local non-specific immunity that might inactivate the infection strain. Accordingly, use of the same route for vaccination and infection might falsely lead to high protection values for the vaccine. A cohabitation model is therefore preferable to an injection model for vaccine testing.

In the cohabitation trial, the strain F272-5 induced cumulative mortalities in the range 34 – 60 %. This is suitable for vaccine testing, although higher values than 34 % are desirable. The strain F272-2 only induced a maximum of 9 % cumulative mortality among cohabitants, and this is too low.

The infection strains were also re-isolated from nearly all diseased cohabitants, and the bacteria were found in tissue lesions. Similar to the previous trial, most survivors were negative for atypical *A. salmonicida*. This indicates that experimental infection of ballan wrasse with atypical *A. salmonicida* will not lead to a chronic infection in survivors. If this also applies to natural infections remains to be shown.

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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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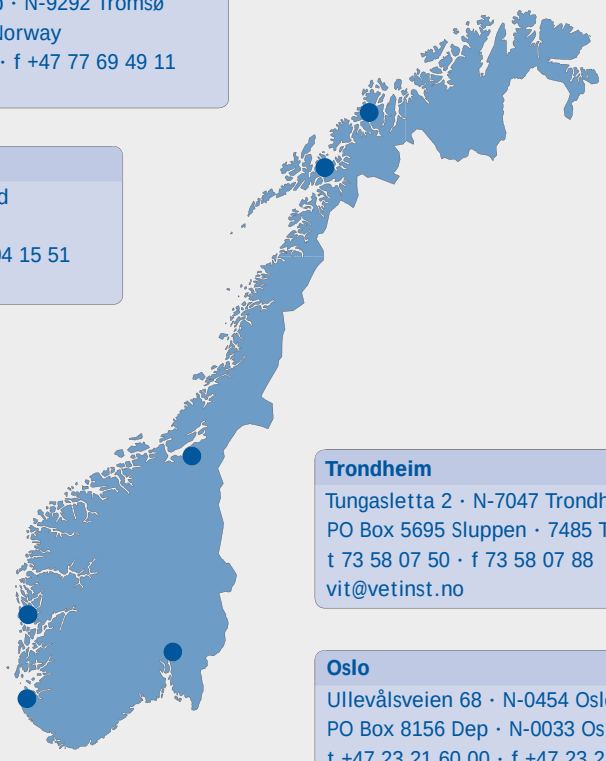

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