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Efficacy and safety of an inactivated vaccine against Salmonid alphavirus (family *Togaviridae*)

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ABSTRACT

Pancreas disease (PD) in salmonid fish is caused by an infection with Salmonid alphavirus (SAV) and remains as one of the major health problems in the European fish farming industry. Sequence studies have revealed a genetic diversity among viral strains. A subtype of SAV (SAV3) is causing an epizootic in farmed salmonids in Norway. Here we evaluate efficacy and safety of an inactivated virus vaccine based on ALV405, a strain of SAV3 that was isolated from Norwegian salmon. The vaccine provided an average relative percent survival (RPS) of 98.5 in an intraperitoneal challenge model, and induced nearly total protection against PD in a cohabitant challenge model. It provided significant protection against SAV-induced mortality also in a field trial under industrial conditions. Local reactions seen as melanization and adhesions in the visceral cavity were less severe than those induced by two commercial vaccines. Finally, we demonstrated that the protection is not impaired when the ALV405 antigen is combined with other viral or bacterial antigens in a polyvalent vaccine. The results confirm that efficient and safe protection against SAV infection and development of PD is possible using an inactivated virus vaccine, both alone and as a component in a polyvalent vaccine.

1. Introduction

Pancreas disease (PD) in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) is caused by strains of the Salmon Pancreas Disease virus (family *Togaviridae*), commonly named Salmonid alphavirus (SAV) [1,2]. The disease has been reported from farmed fish in most European countries that farm salmonids [3]. In Norway, it is regarded as one of the most costly diseases to the industry. The economical loss from PD is a result of several factors including mortality of infected fish, reduced growth of survivors and reduced quality of the fillet [4]. PD is also a welfare problem, since large parts of the fish that are put to sea in Norway become infected.

The genome of SAV is a capped and polyadenylated single-stranded RNA molecule with two open reading frames, encoding non-structural and structural polyproteins [2]. A neutralizing epitope has been mapped to the E2 protein, which functions in receptor-binding in other alphaviruses [5]. Phylogenetic analyses of the partial coding region of E2 have suggested four distinct clades to exist. These clades have been divided into six genetic subtypes, SAV1-6 [6]. The phenotypic consequences of these genetic differences are not known.

The phylogeographic structure of SAV suggests that several independent epizootics of PD are currently occurring in European aquaculture. Most strains from Norway belong to subtype 3 and constitute a distinct epizootic compared to outbreaks in other parts of Europe where subtypes 1, 2, 4–6 have been reported [6–9].

Although wild reservoirs and transmission patterns of SAV are largely unknown, viral RNA has been detected in the water during viraemia, and cohabitant fish are readily infected [1,10]. It therefore appears likely that the virus transmits by water contact once it has entered a farm. Following infection, viral RNA can be detected in most organs of the fish, at least during viraemia. Heart tissues contain the highest levels of viral RNA [3,11]. Tissue lesions have been reported primarily from exocrine pancreas, the heart and skeletal muscle. Lesions in brain and kidney are also found sporadically [3]. The infection may lead to mortality and highly variable mortality rates have been reported from field outbreaks [12,13]. The reason for the variations in mortality rates is not yet understood, but is likely to be a combination of virulence differences among strains of SAV, co-infections with other pathogens and environmental factors.

It is possible to obtain immunity against SAV and several vaccine concepts have been explored [14–17]. An inactivated whole-virus vaccine based on the Irish type-strain of SAV, F93-125 (subtype 1),

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has been commercially available since 2002. Although the industry has vaccinated most fish that are put to sea in the region of Norway where SAV3 is regarded to be enzootic, PD has remained as one of the major disease problems [13]. We have developed an inactivated vaccine based on a strain of SAV subtype 3 – ALV405. Here we evaluate the efficacy and safety of this vaccine, and demonstrate that it could be an attractive new tool for controlling SAV epizootics.

2. Materials and methods

2.1. Vaccines

All vaccines used in this study were water-in-oil formulations where the water phase (containing antigens) was dispersed into a mineral oil phase (continuous phase containing emulsifiers and stabilizers). Emulsification of the antigen with adjuvant was done using a homogenizer with a standard emulsification stator/rotor connected to an emulsion screen. The formalin-inactivated ALV405 antigen was formulated into a monovalent vaccine (ALPHA JECT micro®1 PD, PHARMAQ AS, Norway), or into several polyvalent vaccines where six components that are heterologous to SAV also were present at a fixed concentration, and where the concentration of ALV405 varied as described below. The six additional components were identical to those found in the commercial injectable oil-based vaccines ALPHA JECT micro®6 (0.05 ml/fish dose) and ALPHA JECT®6-2 (0.1 ml/fish dose) (PHARMAQ AS, Norway). These vaccines contain five bacterial (*Aeromonas salmonicida*, *Listonella anguillarum* serotypes 1 and 2, *Vibrio salmonicida*, *Moritella viscosa)* and one viral antigen (infectious pancreatic necrosis virus, IPNV). A vaccine was also formulated without any antigen to serve as an adjuvant placebo control. A commercially available vaccine against SAV (Norvax®Compact PD, MSD Animal Health), was used as reference to the new ALV405-based vaccine in some efficacy studies. Commercial vaccines were always used within the defined expiry date and according to manufacturer recommendations, except that they in lab trials were removed from the original container and transferred by standard sterile techniques to sterile 50 ml tubes that were blinded to the operator.

2.2. Genetic characterization of virus strains

Three different SAV strains were used either as vaccine antigen (ALV405) or as challenge strains (ALV407 or ALV413). These strains originated from Atlantic salmon from Norway diagnosed with Pancreas disease. The genotype of these isolates was determined by sequencing of a 1.3 kB cDNA fragment covering the partial open reading frame encoding structural proteins as previously described [7]. All isolates were confirmed to share >99.8% nucleotide identity to the previously reported SAV3 sequence DQ122130.

2.3. Fish vaccination in laboratory trials

Fish handling, including vaccination, sampling, mortality registration, sample processing and sample analyses was done blinded to the operator. Unvaccinated Atlantic salmon (*S. salar* L.) were sedated using Metacaine (MS222, PHARMAQ Ltd, UK), tagged for identification and vaccinated by intraperitoneal injection. Vaccination was always performed according to the recommendations of the manufacturer and temperature was set to 12 °C, unless otherwise stated. Tanks were monitored daily for clinical signs of disease or mortalities.

2.4. Challenge in laboratory trials

In efficacy trials, fish were challenged with a SAV-strain heterologous to the vaccine strain. Fish were starved 24 h prior to challenge. On the day of challenge, the fish were anaesthetized with Metacaine and i.p. injected with 0.1 ml of the challenge strain. No mortality or abnormal behaviour was observed associated with the challenge procedure.

2.5. Intraperitoneal and cohabitant challenge – efficacy studies

Atlantic salmon ($n = 80$ per group) were tagged by ink tattooing or shortening of adipose fins or maxillae, and vaccinated (mean weight at vaccination: 37.5 g) with one of four vaccines: the monovalent ALV405-based vaccine (0.05 ml/fish), a commercial monovalent SAV vaccine (0.1 ml/fish), a placebo adjuvant vaccine (0.1 ml/fish) or PBS (0.1 ml/fish). After a six weeks smoltification period, the fish were distributed to duplicate tanks with seawater. The fish that were to be evaluated in the i.p. injection model, and that served as shedders for the fish in the cohabitation model, were then challenged with the isolate ALV413 at a final dose of 1.15×10^8 $TCID_{50}$/fish.

Samples from heart, pancreas and skeletal muscle were taken for histological analysis from all cohabitant groups 3–5 weeks post challenge ($n = 10$ per tank/20 per group, per time point, unless otherwise stated). Heart-tissues were also stored on RNA-later (Ambion) and used for RNA extraction and PCR analyses. Sera were collected from the caudal vein for evaluation of viraemia by isolation of infectious virus in Chum salmon heart (CHH) cells using previously described techniques [18,19]. Samples were also taken from surviving fish in the i.p. challenged groups four weeks p.i. ($n = 5$ per tank/10 per group, except for in the PBS placebo group where $n = 4$ and 2 from the two tanks due to few survivors).

2.6. Histology

Tissues were fixed in 10% phosphate-buffered formalin for a minimum of 48 h prior to being submitted blinded to the Norwegian Veterinary Institute, Oslo, Norway for embedment in paraffin wax, sectioning at 4–5 μm and staining with hematoxylin and eosin according to their standard procedure. Blinded slides were scored for lesion severity using a visual analogous scale as previously described [17] (Supplementary Table 1).

2.7. RNA extraction, reverse transcriptase Real-Time PCR and quantification of RNA

Heart samples were collected aseptically without penetrating the peritoneal cavity, stored on RNAlater and submitted to an accredited commercial laboratory for RNA extraction and Real-Time PCR analyses (PatoGen Analyse AS, Ålesund, Norway). The returned results were treated as positive/negative, or semi-quantitative. In the latter case, raw *Ct*-values that were obtained with a previously described Taqman assay targeting the coding sequence of SAV nsP1 [20] were normalized against the *Ct*-values from an assay targeting the mRNA of cellular elongation factor 1a [21] using the Q-gen software [22]. PCR efficiencies for the two assays were provided by PatoGen Analyse AS for inclusion in the analysis (slopes = −3.25 for SAV and −3.41 for ElA). Normalized *Ct*-values were divided by the lowest value in the groups compared and Log2 transformed for presentation.

2.8. Field trial

The trial included two cages of Atlantic salmon (Cage 1: $n = 109\,203$, cage 2: $n = 126\,254$), held under industrial conditions at a commercial seawater fish farm in Western Norway. All the fish were of the same strain and origin and were vaccinated in the freshwater stage (January 11th–February 3rd, 2011) with the commercial multi-component vaccine ALPHA JECT micro®6, that

does not contain any SAV antigens. In addition to this vaccine, all fish were vaccinated with either the commercial monovalent vaccine against SAV or the monovalent ALV405-based SAV vaccine. Vaccines were only injected once in each fish, with a dose of 0.05 ml for ALPHA JECT micro®6 and the ALV405-based vaccine, and 0.1 ml for the commercial SAV vaccine. All vaccinations were done automatically by Lumic vaccination machines (Lumic AS, Norway), according to recommendations from the manufacturers. This implies that fish were vaccinated with the commercial SAV vaccine (December 2nd–14th, 2010) approximately seven weeks prior to injection of ALPHA JECT micro®6, while the ALV405-based vaccine was injected simultaneously with this vaccine. Fish vaccinated with either the commercial SAV vaccine or the ALV405-based vaccine, were held separately until transfer to the sea cages, where they were mixed to avoid cage effects. The proportion of fish vaccinated with the ALV405-based vaccine was 18.3% and 16.1% in cages 1 and 2, respectively, while the remaining fish were vaccinated with the commercial SAV-vaccine. The groups were identified by removal of the adipose fin for fish vaccinated with the ALV405-based vaccine. Mortalities were recorded daily, and fish health was monitored by an external fish health service. Official diagnosis of PD was made by the Norwegian Veterinary institute according to their criteria. Mortalities in the study-population were recorded daily until October 5th, 2011.

2.9. Safety study

Atlantic salmon (mean weight: 35.5 g) were vaccinated with the monovalent ALV405-based vaccine (0.05 ml dose) or the commercial vaccines ALPHA JECT micro®6 (0.05 ml dose) or ALPHA JECT®6-2 (0.1 ml dose) ($n = 35$ in each group). Fish were kept at 17 °C water temperature throughout the experiment. Adhesions and melanization of the viscera were recorded 6 and 12 weeks post vaccination ($n = 15$ per group, per sampling) using a modified Speilberg scale [23].

2.10. Dose-response and efficacy of polyvalent vaccines

The efficacy of polyvalent ALV405-based vaccines with different antigenic dose were tested in a intraperitoneal challenge model. Atlantic salmon were tagged, vaccinated and allocated to duplicate tanks according to Table 1. The challenge was done as described above, except that no cohabitant groups were included, and the challenge isolate ALV407 was used. Efficacy was measured by relative percent survival.

2.11. Statistical analyses

The softwares GraphPad Prism 5 and InStat 3 were used for all statistical analyses. Relative percent survival (RPS) was calculated by the following formula: $(1 - (\%\text{ mortality in test group}/\%\text{ mortality in control group})) \times 100$.

3. Results

3.1. The ALV405-based vaccine protects against SAV-induced mortality and disease following intraperitoneal challenge

The challenge isolate ALV413 caused an accumulated mortality of 87.5% in both parallel tanks in the i.p. challenged fish that had received the PBS placebo vaccine (Fig. 1A). The inactivated ALV405-based vaccine provided a highly efficient protection against mortality with a relative percent survival of 100 and 97 in the two parallel tanks (average RPS = 98.5). It performed significantly better than the commercial SAV vaccine, which gave an RPS of 79 and 51 (Average RPS = 65, $p < 0.0001$ using Fisher's exact test). The adjuvant placebo vaccine also provided a significant effect compared to the PBS placebo group, with an RPS of 45 and 20 (average RPS = 32.5, $p < 0.0001$ using Fisher's exact test).

Virus RNA levels in hearts were measured four weeks p.i. in five surviving fish per tank per group. This demonstrated that viral RNA was efficiently produced in all groups except the groups vaccinated with the inactivated ALV405-based vaccine (Fig. 1B). In these latter groups, fish seemed to be completely protected against replication of the challenge strain. Viral RNA production in survivors did not differ in this organ between the placebo-vaccinated groups and the groups vaccinated with the commercial SAV vaccine. Similarly, histopathological changes developed in heart, pancreas and skeletal muscle of all groups except in the groups vaccinated with the ALV405-based vaccine (Fig. 1C).

3.2. Protection against development of PD in a cohabitation challenge model

No significant mortality was obtained in the cohabitation model and efficacy was therefore evaluated by quantification and prevalence of infectious virus particles in serum, viral RNA in heart tissue and histological lesions in heart, pancreas and skeletal muscle.

Accumulated prevalences of infectious virus in sera sampled throughout the experiment were determined in groups vaccinated with ALV405-based vaccine, commercial SAV vaccine, Placebo Adjuvant and Placebo PBS to be 2%, 23%, 35% and 39%, respectively. The qualitative assessment of histological changes demonstrated full development of PD in all groups except for the groups vaccinated with the ALV405-based vaccine. The accumulated prevalence of fish carrying viral RNA was higher than 90% in all groups except for those vaccinated with the ALV405-based vaccine (Fig. 2A). Total prevalences of pancreatic lesions that accumulated throughout the study in the PBS and Placebo Adjuvant groups were 91.5% and 90%, respectively. In the groups vaccinated with the ALV405-based vaccine and the commercial SAV vaccine, the prevalences were 3.2% and 80% ($n = 60$ in each group, except the PBS group where $n = 59$).

Quantitative differences between the ALV405 vaccinated fish and the other groups were found to be significant (One-way ANOVA with Bonferroni's multiple comparison test) both when comparing levels of viral RNA (Fig. 2B) and histological scores in heart tissues, pancreatic tissues and skeletal muscle (Fig. 3A–D). No significant differences were found when comparing the three other groups.

3.3. The ALV405-based vaccine reduces SAV-induced mortality in a field outbreak

The efficacy of the ALV405-based vaccine was tested under field conditions at a commercial farm. Fish had been vaccinated with either the ALV405 vaccine or the commercial SAV vaccine, tagged and kept in the same netpen to avoid cage-effects. Under these conditions, a PD outbreak was officially diagnosed by histopathological and PCR analyses. The ALV405-based vaccine reduced mortality significantly ($p < 0.0001$, Chi-square test) compared to the commercial SAV vaccine, from 8.4% to 5.6% in cage 1 (Fig. 4A) and 19.2% to 8.2% in cage 2 (Fig. 4B).

3.4. The ALV405-based vaccine is safe

Local reactions of the ALV405-based vaccine were evaluated six and twelve weeks after vaccination. The vaccine induced less adhesions (Fig. 5A and B) and melanization (not shown) of the viscera than the commercially available vaccine ALPHA JECT®6-2 both when injected alone, and when injected together with the six-component vaccine ALPHA JECT micro®6.

Table 1
Efficacy of a 7-component vaccine is dose-dependent. A 7-component test vaccine was made with various amounts of ALV405-antigen in addition to antigen from six other fish pathogens. The relative amount of ALV405-antigen is indicated. Efficacy was measured by RPS compared to fish injected with PBS.

Vaccine	Fish per tank (*n*)	Relative amount of ALV405 antigen	Fish identification[a]	RPS tank1	RPS tank 2
PBS	40	0	None	NA	NA
ALPHA JECT micro® 6	40	0	AF	35	63
6-components + 1× ALV405	40	1	LM + AF	64	66
6-components + 50× ALV405	40	50	RM + AF	97	92
6-components + 100× ALV405	40	100	LM	89	96
6-components + 200× ALV405	40	200	RM	97	96

[a] Fish were tagged by shortening of right maxillae (RM), left maxillae (LM) or the adipose fin (AF).

3.5. Protection is dose-dependent and reproducible in a seven-component vaccine formulation

The ALV405 antigen was formulated with four different doses into a polyvalent vaccine containing six components from heterologous fish pathogens. Vaccination of fish in a laboratory trial with these polyvalent vaccines demonstrated an RPS of 97 and 96 in the parallel tanks at the highest dose (Table 1). When the dose was reduced 200-fold, the RPS was 64 and 66, demonstrating a dose-response effect.

4. Discussion

This study is the first description of the performance of a SAV vaccine under laboratory and field conditions. Most vaccines against bacterial fish diseases are based on inactivated bacteria, and are generally accepted to induce strong immunity [24]. Vaccines for finfish based on inactivated viruses have also been developed, but their protection is often limited to the reduction of disease severity, more than a complete protection against disease [25]. Previous attempts to immunize fish with inactivated SAV have indicated that it is possible to obtain some protection in laboratory trials [14,17,16]. Here we have demonstrated that an inactivated vaccine that is based on the Norwegian SAV3 strain ALV405, has a safety profile equal to or better than existing commercial vaccines, and can provide a highly efficient protection against infection with SAV and subsequent development of PD. We also demonstrated the attractive possibility of including the ALV405 antigen in a seven-component vaccine.

Efficacy of the vaccine was tested in three fundamentally different challenge models in order to obtain a realistic picture of its performance. The monovalent ALV405-based vaccine induced close

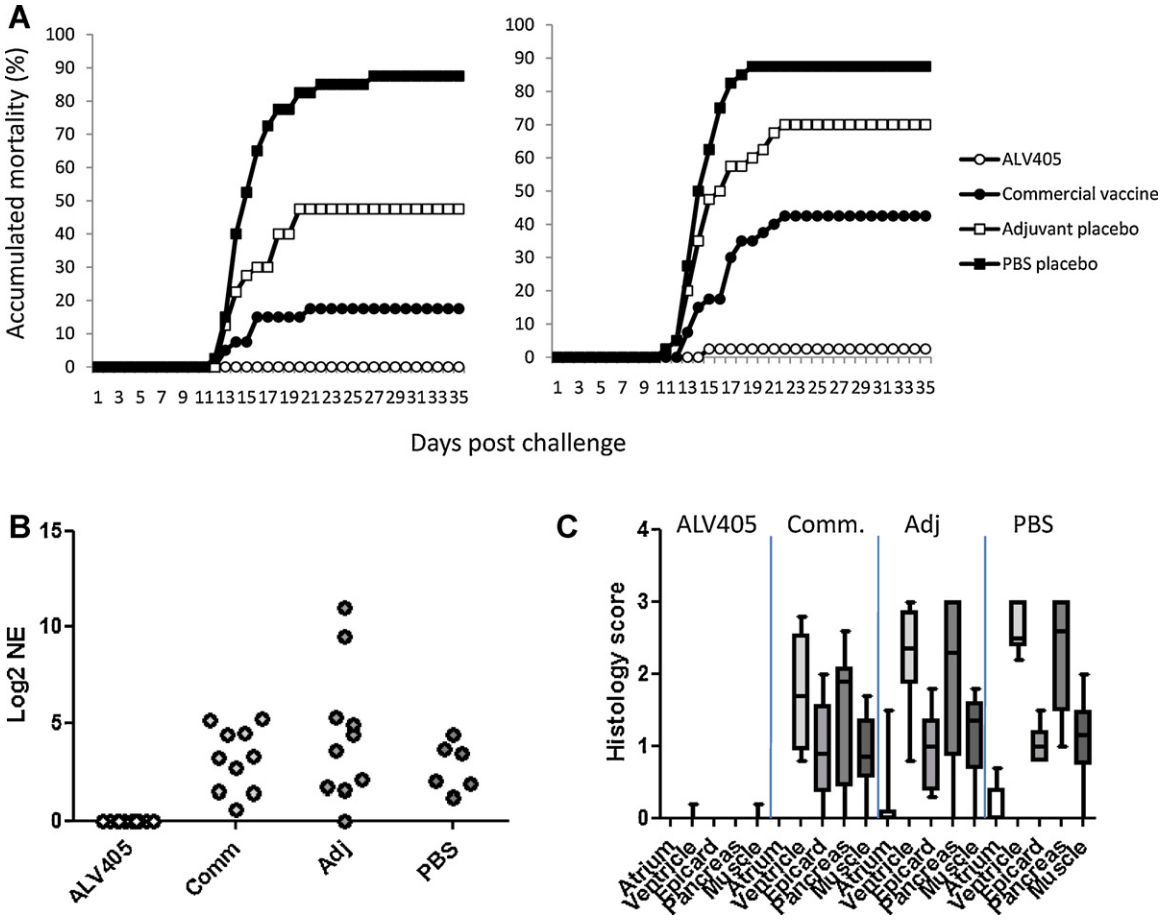

Fig. 1. Protection against SAV induced mortality of different vaccines in an i.p. challenge model. (A) The experiment was run in two parallel tanks and accumulated mortality curves for both tanks are shown. (B) Normalized expression of viral RNA in heart of survivors sampled four weeks p.i. from both parallel tanks (data combined for the sake of presentation). Fewer fish were sampled in the PBS group due to few surviving fish. (C) Quantitative histological assessment of heart (atrium, ventricle and epicard), pancreas and skeletal muscle in the same fish as those presented in (B). ALV405 = ALV405-based vaccine, Comm. = commercial vaccine, Adj. = placebo adjuvant, PBS = placebo PBS.

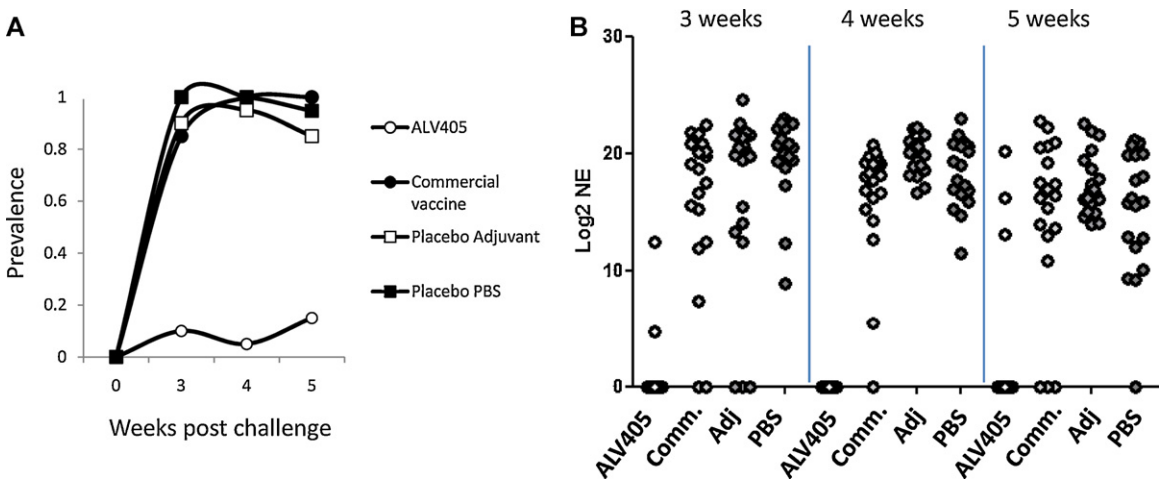

Fig. 2. Prevalence and quantification of viral RNA in hearts of fish challenged by cohabitation. (A) The prevalence of fish with detectable levels of viral RNA in heart tissues, as measured by Real-Time RT PCR. (B) Log2 transformed normalized expression of viral RNA in hearts of all sampled individuals. Duplicate tanks were used with similar results. Results from both tanks are combined for the sake of presentation ($n = 20$ per group per time-point). ALV405 = ALV405-based vaccine, Comm. = commercial vaccine, Adj. = placebo adjuvant, PBS = placebo PBS.

to complete protection against replication, histopathology and mortality in both i.p. and cohabitation models, and fish were significantly protected against mortality in a field trial under industrial conditions. Results from a second farm where the ALV405-based vaccine has been used are in concordance with those shown in the present work. We have however observed that vaccinated fish surviving a field outbreak, show histological signs of PD. A likely explanation for a potentially reduced performance in the field compared to what is seen in laboratory trials is the constant presence of various heterologous pathogens in field populations. In the farm included in this study, as well as in the second farm described above, at least two other pathogens, sea lice (*Lepeophtheirus salmonis*) and the microsporidian *Paranucleospora theridion*, were present in the fish population. Both parasites are common in farmed populations of Atlantic salmon in Norway, and believed to have immune-suppressive effect on the host [26,27].

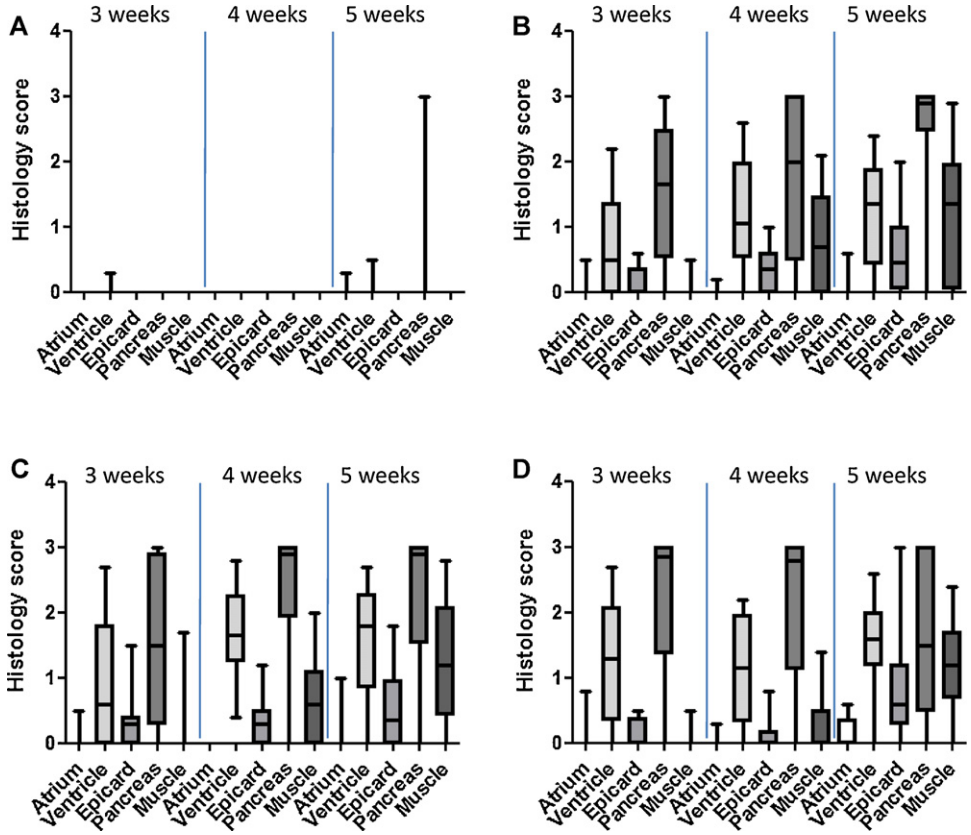

Fig. 3. Quantification of tissue lesions in vaccinated fish challenged by cohabitation. Fish were vaccinated with the ALV405-based vaccine (A), a commercial vaccine (B), a placebo adjuvant vaccine (C) or PBS (D), and the histological lesions that developed 3–5 weeks post challenge are shown ($n = 20$ per time-point, per group).

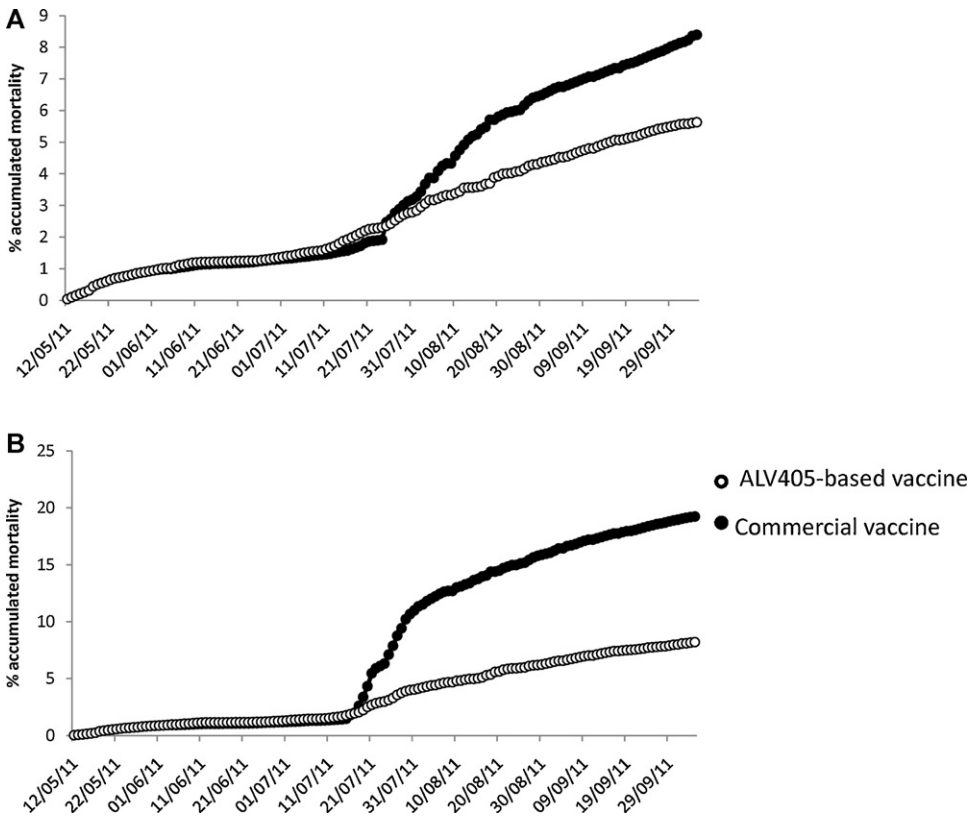

Fig. 4. Efficacy of the ALV405-based vaccine under industrial field conditions. A commercial fish population was vaccinated in the period December 2010–February 2011 with either the ALV405-based vaccine or a commercial SAV vaccine, and kept as a mixed population in two cages (A and B). Fish vaccinated with the ALV405-based vaccine were tagged by adipose fin removal for identification. The farm experienced an outbreak of PD, and the accumulated mortalities after sea transfer from both cages are shown. Differences in accumulated mortality among test groups are significant ($p < 0.0001$) according to a Chi-square test.

Although the efficacy of the ALV405-based vaccine was similar in both laboratory models used, the efficacy of the commercial vaccine and the placebo adjuvant vaccine was not. Both vaccines appeared to provide a significant effect in the i.p. challenge model that could not be detected when fish were challenged through the assumed natural challenge route, i.e. in the cohabitation model. The conflicting results observed for the two laboratory models are likely to result from the fact that the challenge virus is injected in the same spatial area as the vaccine in the i.p. model. Thus the challenge virus is released into an area where there is a chronic and active inflammatory response [28]. These results highlight the importance of studying vaccines under various conditions to obtain a more complete understanding of their performance.

The present vaccine situation in the European salmonid farming industry is suboptimal. Despite vaccination of the fish population in exposed areas, the SAV epizootics remain as a major loss-contributing factor to the industry [4]. Moreover, the available SAV-vaccine must be given as a separate injection from a multi-component vaccine, with at least 230 day degrees separating the injections. This is an additional stressor for the fish and costly to the farmer. The high level of protection combined with the possibility to include the ALV405 antigen in a multi-component vaccine could

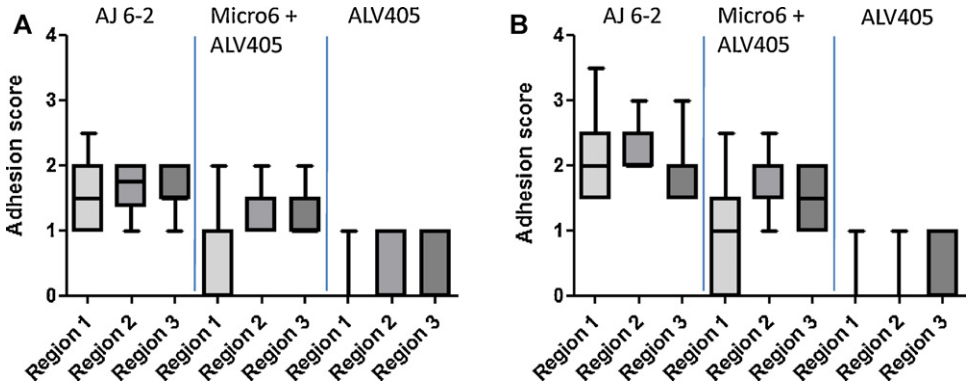

Fig. 5. Safety profile of the ALV405-based vaccine compared to commercial vaccines. Local reactions defined by adhesions of the viscera, were scored according to a modified Speilberg's scale 6 (A) and 12 weeks (B) post vaccination in three different regions of the peritoneal cavity. AJ6-2 = ALPHA JECT® 6-2, Micro6 = ALPHA JECT micro® 6, ALV405 = monovalent ALV405-based vaccine.

therefore represent a significant improvement for both fish health and farming economy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.05.069.

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