ARTICLE IN PRESS

Fish and Shellfish Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Protection and antibody reactivity in lumpsucker (*Cyclopterus lumpus* L.) following vaccination against *Pasteurella* sp.

Rebecca Marie Ellul[a,*], Jan Bulla[b,c], Espen Brudal[d], Duncan Colquhoun[e], Heidrun Wergeland[a], Anita Rønneseth[a]

[a] Department of Biological Sciences, University of Bergen, P.O. Box 7803, N-5006, Bergen, Norway
[b] Department of Mathematics, University of Bergen, P.O. Box 7803, N-5006, Bergen, Norway
[c] Department of Psychiatry and Psychotherapy, University Regensburg, Regensburg, Germany
[d] PHARMAQ AS, Harbitzalléen 2A, P.O. Box 267 Skøyen, N-0213, Oslo, Norway
[e] Norwegian Veterinary Institute, Oslo, Norway

ARTICLE INFO

Keywords:
Antibodies
Challenge
Immunology
Infection
Lumpsucker
Pasteurellosis
Pathology
Vaccination

ABSTRACT

Two monovalent vaccines against pasteurellosis were developed and tested for efficacy using a previously established bath challenge model. High levels of specific antibodies were detected following vaccination. While the vaccine efficacy trial indicated that some level of protection was obtained, high mortality was still observed. qPCR analysis of head kidney tissues from surviving fish post challenge showed no difference in bacterial numbers in vaccinated and non-vaccinated fish. Clinical symptoms observed in moribund and diseased fish included white spots on the skin and around the eyes, frayed fins and redness around the mouth and fin bases. Despite production of specific antibodies, the protection against experimental challenge was relatively weak. A reason for this could potentially be that the specific antibodies produced are not alone enough to provide complete protection against pasteurellosis in lumpsuckers. Confocal and scanning electron microscopy of head kidney leucocytes exposed to *Pasteurella* sp. *in vitro* gave indications of the interactions between the pathogen and leucocytes. The results indicate that parts of the immune system other than humoral antibodies could be important for protection against pasteurellosis. Our combined results highlight the need for further work on host-pathogen interaction between *Pasteurella* sp. and lumpsuckers.

1. Introduction

Lumpsucker (*Cyclopterus lumpus* L.) is a marine species of cleaner fish used for biological control of sea lice infecting farmed Atlantic salmon. In Norway, wild populations as well as farming operations can be found all along the entire coastline, from Troms county in the north, to Vest-Agder county in the south. Due to their ease of culture and relatively high delousing activity at lower water temperatures, lumpsuckers are ideally suited and preferred over various species of wrasse for use as cleaner fish. For this reason, the demand for farmed lumpsucker has been steadily increasing, with approximately 40 million fish used in Atlantic salmon production in Norway in 2018 [1].

As a relatively new species to aquaculture, several bacterial diseases have been documented for farmed lumpsuckers. The most commonly encountered bacterial agents from outbreaks are *Vibrio* species (including *V. anguillarum* and *V. ordalii*), *Tenacibaculum* spp., *Moritella viscosa*, *Pseudomonas anguilliseptica*, atypical *Aeromonas salmonicida* and an as yet unnamed *Pasteurella*-like species [2–4].

Outbreaks of pasteurellosis have been steadily increasing since the first case was recorded in 2012, with 28 sites reporting outbreaks in 2016 [5]. Despite the decrease by half in reported cases to 14 in 2018 [1], *Pasteurella* sp. remains a problematic pathogen for fish farmers, and under-reporting of outbreaks cannot be excluded. The bacteria affect all life stages of lumpsucker, and to date there is no adequate treatment for this disease. The *Pasteurella* sp. pathogenic for lumpsucker is closely related to, albeit serologically distinct from, *Pasteurella skyensis* [6], when analysed using rabbit antisera [3]. It is also phenotypically similar, but genetically distinct from a group of *Pasteurella* isolates pathogenic for farmed Atlantic salmon in Norway, which causes “varracalbmi”, a systemic infection characterised by severe ophthalmitis [7].

Prophylactic measures including vaccines have been commercially developed for several lumpsucker diseases. Currently, the majority of farmed lumpsucker are vaccinated against *V. anguillarum* and atypical *A. salmonicida*. As *Pasteurella* sp. is a relatively recently discovered

* Corresponding author.
E-mail address: rebecca.ellul@uib.no (R.M. Ellul).

https://doi.org/10.1016/j.fsi.2019.11.016
Received 16 September 2019; Received in revised form 30 October 2019; Accepted 5 November 2019
1050-4648/ © 2019 Elsevier Ltd. All rights reserved.

pathogen, and is difficult to culture in liquid media, vaccine development against pasteurellosis remains at an early stage. A *Pasteurella* component is, therefore, not currently included in commercial lumpsucker vaccines.

Despite their widespread use, knowledge of the lumpsucker immune system remains limited [8]. Haugland et al. (2012) first characterised the components and functionality of the lumpsucker innate immune system by isolating leucocytes from peripheral blood, spleen and head kidney, and determined the phagocytic capacity of lumpsucker leucocytes to be very high. Rønneseth et al. (2015) [9] then characterised B cells from lumpsuckers and found high phagocytic ability among IgM$^+$ B cells isolated from blood. Further, it was found that immunisation resulted in the production of specific antibodies and vaccine-induced protective immunity against experimental challenge with atypical furunculosis. In previous work [10], we confirmed that *Pasteurella* sp. causes significant mortalities in lumpsucker, and determined that a bath challenge model is the most adequate to study progression of the disease.

In the current study, we investigated the adaptive immune response of lumpsucker following immunisation against, and subsequent exposure to *Pasteurella* sp.. We also studied the effects of *in vitro* exposure of isolated lumpsucker head kidney leucocytes to *Pasteurella* sp. through immunofluorescence and confocal microscopy, as well as scanning electron microscopy. This was done to shed light on the mechanisms of infection of the pathogen, and in turn clarify how the lumpsucker immune system needs to be stimulated to prevent infection and disease.

2. Materials and methods

2.1. Bacterial isolates and culture

The *Pasteurella* sp. isolate described in previous studies [3,10] was identified from a natural *Pasteurella* sp. outbreak in lumpsucker, and was used in this work for vaccine preparation, challenge and *in vitro* exposure of leucocytes. Briefly, bacteria were grown in tryptic soy broth (TSB) (Becton Dickinson supplemented with 1.5% NaCl and 10% foetal bovine serum (Gibco, Lot no. 1739464) at 20 °C with shaking (200 rpm). For challenge, the cultures were harvested in the late exponential growth phase and centrifuged at 2500 *g* (Beckman Coulter Allegra X-15R) for 15 min at 4 °C. Cells were washed once with sterile phosphate buffered saline (PBS) (Lonza, Lot no. 8MB014), followed by centrifugation and resuspension in PBS prior to use.

2.2. Vaccine preparation and vaccination

The vaccines were formulated as water-in-oil emulsions by PHARMAQ AS, Norway. Two different monovalent vaccine preparations were tested, both based on formalin killed *Pasteurella* sp. emulsified in adjuvant. Prior to inactivation, bacterial cell numbers were measured using a cell counter (CASY Model TT (Innovatis) and CASY worX V1.26) to be approximately 2×10^9 bacteria mL^{-1} and 150 mL culture volumes were used to prepare the vaccines. The antigen concentration in one of the vaccines was concentrated by centrifuging a formalin-killed culture of bacteria and removing 90% of the supernatant. The other vaccine was not concentrated. In addition, control groups were vaccinated using a monovalent *V. anguillarum* O1 vaccine (control vaccine) and phosphate buffered saline (PBS), respectively.

Vaccination was performed by intraperitoneal injection of vaccine (50 μL) using Socorex self-refilling syringes. The antigen dose per fish was $> 2 \times 10^7$ and $> 2 \times 10^8$ cells fish^{-1} for the non-concentrated and concentrated vaccine respectively. Vaccination was carried out six weeks before bacterial challenge. At the time of vaccination, the fish had an average length and weight of 5.1 ± 0.3 cm (n = 50) and 9.8 ± 1.8 g (n = 50) respectively. Vaccine groups were identified by Visual Implant Elastomer (VIE tags, Northwest Marine Technology Inc.) placed subcutaneously on the forehead.

2.3. Fish and rearing conditions

Farmed, unvaccinated lumpsuckers were obtained from Vest Aqua Base AS, Norway. The experimental population had a disease free history, displayed no signs of infection or mortality and were screened and certified free from atypical *A. salmonicida*, *Pasteurella* sp., *Vibrio anguillarum* and lumpfish flavivirus (PHARMAQ Analytic). The fish were acclimated for 15 days at 12 °C in 500 L tanks at a photoperiod of 12 L: 12D, a salinity of 34 ppt and oxygen saturation of > 77% in the outlet water, at the holding facilities in the Industrial and Aquatic Laboratory (ILAB) at the Bergen High Technology Centre. The fish were fed Amber Neptun (Skretting) dry feed by automatic feeders and fasted for 36 h prior to vaccination and challenge. The fish were then transferred to the challenge unit 3 days prior to the challenge and split into 150 L tanks, with 100 fish per tank. The same water parameters were used as for the holding tanks.

2.4. Lumpsucker immune sera

Blood samples were collected post-vaccination from individual fish vaccinated with the test vaccines every 100° days for a period of 600° days and stored overnight at 4 °C. Sera were then obtained by centrifugation at 1300*g* and stored at −20 °C.

2.5. Quantification of lumpsucker-specific antibodies to Pasteurella sp.

Analysis for specific antibody production was carried out by enzyme-linked immunosorbent assay (ELISA). Freeze-dried *Pasteurella* sp. was used as the antigen to coat 96-well plates (Nunc). The antigen was dissolved in 0.01 M PBS, pH 7,3 and sonicated for 2 min at 20 kHz and diluted to a concentration of 10 μg mL^{-1} before being used. Lumpfish sera were diluted (1:50) in PBS containing 0.05% Tween 20. Specific antibodies were detected using rabbit anti-lumpfish IgM serum (1:1500) [4] and peroxidase conjugated goat anti-rabbit immunoglobulin (Dako) (1:2000).

The optical density (OD) was read at 492 nm in a Sunrise microplate reader (Tecan Group Ltd.) using Magellan software. Each dilution was carried out in parallels of two. The blank control employed was wells containing PBS instead of lumpfish serum. For an internal positive control, serum from lumpfish immunised with *Pasteurella* sp. antigens used by Ref. [9] was included.

Agglutination tests were performed using sera from lumpsuckers vaccinated against *Pasteurella* sp. in this work. The tests were carried out by inoculating live *Pasteurella* sp. onto glass slides, followed by the addition of the sera and observing for flocculation. Serum from lumpsucker triple immunised against *Pasteurella* sp [9]. were used as a positive control, while pre-vaccination serum from this study was used as a negative control.

2.6. SDS-PAGE silver staining and western blots of Pasteurella sp.

Whole protein profiles were analysed by SDS-PAGE (12% acrylamide) according to established methods [11] with minor modifications as described previously [12]. Electrophoresis was performed using a Mini Protean Tetra Cell (Bio-Rad). *Pasteurella* sp. prepared as described in Section 2.5 was heat-treated (96 °C for 5 min) in sample-buffer containing β-mercaptoethanol. Samples of 10 μL were loaded onto each well, electrophoresed at 190 V for 45 min, followed by staining of proteins using Silver Stain Plus kit (Bio-Rad) [13].

Western blotting was performed using whole bacteria after fractionation on a 12% SDS-polyacrylamide gel as described above and electro-blotted onto 0.45 mm nitrocellulose membranes (Bio-Rad) as described in Ref. [14] with some minor modifications. Briefly, analysis was performed using serum collected from the vaccinated lumpsucker diluted 1:100, and rabbit anti-*Pasteurella* diluted 1:10,000. Sera from non-immunised fish were used as negative controls while sera from

Table 1
Challenge details showing tank group, total number of fish used and challenge doses. There were two tank parallels for each vaccine group (25 fish per vaccine group per tank).

Tank	Dose (bacteria mL^{-1})	Vaccine group	n fish
1	1×10^5	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50
2	5×10^5	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50
3	1×10^6	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50
4	1×10^7	PBS control	50
		Control vaccine	50
		Non-concentrated vaccine	50
		Concentrated vaccine	50

three times immunised lumpsuckers were also used for comparison [9]. For the production of rabbit and lumpsucker anti-*Pasteurella* immune sera, Freund's Complete Adjuvant and Freund's Incomplete Adjuvant were used respectively. Anti-lumpfish IgM serum was diluted 1:1000, polyclonal goat anti-rabbit immunoglobulins/HRP (Dako) diluted 1:2000. These were developed with HRP Conjugate substrate kit (Bio-Rad) or Clarity Western ECL substrate (Bio-Rad) and visualized in Bio-Rad molecular image chemi Doc XRS þ Imaging system. The molecular weight of the SDS-PAGE fractions and immune reactive bands were identified using SDS-PAGE low range standard (Bio- Rad) and Kaleidoscope pre-stained standard (Bio-Rad), respectively.

2.7. Challenge of vaccinated fish

Six weeks (500° days) post-vaccination fish were bath challenged as described in previous work [10]. Each of the four bacterial doses used for challenge were tested in duplicate, resulting in eight tanks used. Each tank contained 25 fish for each of the two test vaccine groups and control groups, giving 100 fish in each tank (Table 1). Briefly, fish were transferred to aerated static challenge tanks (25 L) containing the respective bacterial doses for 1 h, after which they were returned to clean holding tanks. The fish were then checked twice daily and dead and moribund fish removed accordingly.

Confirmation of infection was performed by re-isolation of bacteria from head kidney samples on blood agar (with 2% NaCl) from all the dead and moribund fish. Kidney samples from the dead fish were also stored in RNAlater stabilising solution at −20 °C to confirm presence of *Pasteurella* sp. by qPCR analysis.

2.8. Quantitative PCR

The qPCR analysis was performed as described previously [10]. DNA was extracted from head kidney samples using the DNEasy Kit (Qiagen), according to the manufacturer's instructions for animal tissue samples. The amount of *Pasteurella* sp. for each sample was determined by qPCR using SYBR Green (Sigma). The primers used were RK-Past F: 5′-TTCACCATTCAAAGCACCATCAAG-3′ and RK-Past R1: 5′-CTTCTAA AGCAGCATTGGCATTAT-3′ targeting the superoxide dismutase (sodA) gene. Each qPCR reaction contained a volume of 25 μL and consisted of 12.5 μL 2X SYBR, 1 μL each of the forward and reverse primers (10 μM), 0.5 μL of RNAse and DNAse-free water, and 10 μL of genomic DNA (50 ng). A C1000 Touch thermal cycler (Bio-Rad) was used for qPCR, with the following cycle conditions: (1) 94 °C for 5 min (2) 40 cycles of 94 °C for 15 s, 60 °C for 1 min and (3) an increase from 60 °C to 92 °C at a rate of 1 °C/5 s. Data was analysed using Bio-Rad CFX Manager 3.1 software (Bio-Rad).

2.9. Isolation and exposure of head kidney leucocytes to Pasteurella sp.

Six lumpsucker were quickly netted and killed by a sharp blow to the head. Leucocytes were isolated from the head kidney on discontinuous Percoll gradients as described previously [8] with the following modifications. The supplemented L-15 medium did not contain gentamicin sulphate, since the cells were to be exposed to viable *Pasteurella* sp. Additionally, resuspension of the isolated leucocytes was done in L-15 supplemented with 5% foetal calf serum (L-15/FCS). The leucocytes were counted in a CASY-TT Cell Counter TM (Innovatis AG) and viability and aggregation factor determined.

The concentration of the isolated leucocytes was then adjusted to 1×10^7 cells mL^{-1} in L-15/FCS and 500 μL volumes were added to each well of 4-chambered chamber slides (5×10^6 cells per well) (Thermo Scientific Nunc) and incubated overnight at 15 °C. The next morning, non-adherent cells were removed by removing the growth medium.

An overnight culture of *Pasteurella* sp. was centrifuged and re-suspended in L-15/FCS and adjusted to 2×10^5 bacterial cells mL^{-1}. 500 μL volumes were then added to each well (1×10^5 bacteria per well). Sterile L-15/FCS medium was used for the non-challenged controls. The cells were then incubated at 15 °C on a shaking incubator set at 60 rpm for 6 h before washing and supplementing with sterile L-15/FCS medium.

2.10. Immunofluorescence staining of bacterial exposed leucocytes

Leucocytes were sampled at 6 and 24 h post bacterial exposure. The L-15/FCS medium including suspended bacteria was discarded and the cells fixed in 3.7% freshly prepared paraformaldehyde for 10 min. Permeabilisation was carried out using 0.1% Triton-X 100 in PBS (v/v) for 5 min.

The wells were then blocked using 2% BSA in PBS (w/v) for 1 h, then incubated for 1 h in a humidity chamber in the dark with primary antibodies (rabbit anti-*Pasteurella*) diluted 1:10,000 in PBS containing 0.5% (w/v) BSA. This was followed by incubation in darkness with Alexa Fluor™ 555 F(ab')2 fragments of goat anti-rabbit IgG (H + L) (2 mg mL^{-1}) (Invitrogen) diluted 1:400 in PBS containing 0.5% (w/v) BSA for 1 h.

The cells were then incubated in darkness with Alexa Fluor™ 488 phalloidin (Invitrogen) diluted 1:40 in PBS containing 1% (w/v) BSA for 20 min and stained with Hoechst 33342 diluted 1:2,000 in PBS for 10 min before mounting with Vectashield® antifade mounting medium (Vector). Wells were washed three times using PBS between each incubation, and further rinsed with distilled water prior to mounting.

Samples were imaged using a Leica TCS SP8 STED 3X confocal microscope at the Molecular Imaging Centre (MIC), Bergen.

2.11. Scanning electron microscopy

Leucocytes were prepared and infected as described in Section 2.7 and sampled at 6 and 24 h post infection. The medium was then removed, and cells washed three times using PBS. The cells were then fixed in 2% glutaraldehyde diluted in 0.1 M sodium cacodylate for 2 h at 4 °C. These were then washed three times in 0.1 M sodium cacodylate for 15 min. Post-fixation was then done in 1% osmium tetroxide diluted in sodium cacodylate for 1 h. Cells were then washed twice in buffer and dehydrated in an alcohol series as follows. 35%, 50% 70% 96% alcohol each for 20 min, respectively, followed by 100% ethanol, three times for 20 min each.

Samples were then submitted to critical point drying (Quorum K850), mounted on aluminium stubs and sputter coated (Jeol JFC-2300HR) with 10 nm Au/Pd and imaged with a Jeol JSM-7400F (Tokyo, Japan) using 5 KV and LEI detector at the Molecular Imaging

ARTICLE IN PRESS

Centre (MIC), Bergen.

2.12. Statistics

All statistical analyses were carried out using the software R 3.5.1 (R Core Team 2018). In order to avoid false positives, the p values resulting from multiple comparisons carried out on the same data set were adjusted using the method of Benjamini & Hochberg (1995). The level of statistical significance for all analyses was set at $p = 0.05$. The whiskers shown on box plot figures were scaled with factor 1.5, as the default setting in R.

2.12.1. ELISA

The ELISA data is of hierarchical structure, with within-subject repeated measures. Therefore, linear mixed-effects models were used to examine these effects. In order to evaluate which model is most appropriate for the data, various model specifications were considered. Following Pinheiro & Bates (2000), these data were compared using the model selection criteria Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and Likelihood Ratio Tests (LRT). The parameters of the finally selected models were re-estimated via REML. When a significant effect was found, post hoc-type comparisons were performed within the mixed effects framework.

2.12.2. Survival analysis

The results from the vaccine trial were analysed by classical methods from survival analysis, specifically the non-parametric Kaplan-Meier framework. Different Kaplan-Meier curves were compared by the log-rank test. For each bacterial dose used in the bath challenge, a test for an effect of any vaccine type on the hazard function was first carried out. If an effect was found, post-hoc tests were carried out to determine which of the vaccine types was significant.

Differences in mortalities between the non-concentrated and concentrated vaccines at the end of the trial were analysed using Chi square tests for 2×2 contingency tables.

The qPCR data on presence of bacteria in fish sampled from the vaccine trial was $\log_{10}$ transformed and analysed using the one-way ANOVA test.

3. Results

3.1. ELISA

Analyses of sera collected from vaccinated lumpsucker showed that specific antibody production was induced by vaccination and that the levels of antibodies produced increased over time. Significantly different levels of specific antibodies were detected when comparing sera from fish vaccinated with the two test vaccines, where the fish vaccinated with the concentrated vaccine produced significantly higher levels of specific antibodies. For both vaccines, the highest levels of specific antibodies were detected at 500° days post vaccination.

The optical density (OD) of antibody levels induced by vaccines at different degree days are shown in Fig. 1. The OD values increase with time, with a strong effect at 300° days (for the concentrated vaccine) and 400° days (for the non-concentrated vaccine); the OD values for the non-concentrated vaccine seem to rise less steeply than for the concentrated vaccine.

Statistically, the results from post-hoc tests confirmed that the OD values are significantly different to the baseline from 300° days onwards for the concentrated vaccine and from 400° days onwards for the non-concentrated vaccine. Furthermore, from 400° days onwards, OD values for the concentrated vaccine are significantly different from those corresponding to the non-concentrated vaccine.

3.2. Vaccine efficacy trial

A range of bacterial challenge doses was investigated in order to determine the optimal level to test the efficacy of the vaccines being developed. As shown in Fig. 2, the lowest challenge dose resulted in very low mortalities (below 40% in all groups) and for this reason, this dose was not used for further statistical analysis. The other challenge doses all resulted in mortalities higher than 60% for the two control groups. The mortalities from the two vaccine groups follow a similar pattern to each other; however, in the case of the highest challenge dose, mortalities were considerably higher than 60% mortality.

The concentrated vaccine resulted in the best survival rates of all the vaccines tested. However, statistical analysis failed to verify any significant difference in survival rates found between the two *Pasteurella* sp. vaccines tested, at any bacterial dose. For the highest three challenge doses there was a significant difference ($p < 0.05$) in survival rates between the PBS controls and the fish immunised with the concentrated vaccine, overall. The survival rates from the fish immunised with the non-concentrated vaccine differed significantly from those of the control vaccinated fish only in the 1×10^6 and 5×10^5 bacteria mL^{-1} challenge doses.

Chi square analysis of the relative percent survival at the end of the challenge (Table 2) however indicates that a significant difference can be observed between the two vaccines.

Clinical symptoms observed in the challenged fish included those reported previously [10], such as haemorrhage and redness at the base of fins and on the lower jaw, as well as white spots along the body and around the eyes. In addition, frayed tail fins were also encountered, as recorded in other work [3].

The antisera raised against *Pasteurella* sp. in lumpsucker contained antibodies reactive towards proteins of high molecular weight (more than 75 kDa) (Fig. 3 B, C, D). Sera from triple immunised lumpsuckers (Fig. 3D) additionally contained antibodies reactive to proteins of approximately 37 kDa and 48 kDa, which may correspond to major protein bands identified by silver staining (Fig. 3A). The sera from the vaccinated fish also reacted to the protein at approximately 48 kDa, albeit not as strongly as the serum from triple immunised lumpsuckers. The sera from lumpsucker vaccinated with concentrated and non-concentrated vaccine contained antibodies reactive towards proteins of similar molecular weight. The sera produced in rabbit contained antibodies reactive towards several proteins of varying molecular weight, including a band at just below 20 kDa (Fig. 3E) which was not identified by the other sera. By comparison, sera from the triple immunised lumpsucker (Fig. 3D) did not contain the same variety of antibodies as the serum from immunised rabbits. All sera, including the control serum, reacted to components at approximately 15 kDa.

3.3. Re-isolation of *Pasteurella* sp.

Pasteurella sp. was re-isolated from all fish sampled following bath challenge when grown on blood agar containing 2% sodium chloride. qPCR also confirmed the presence of *Pasteurella* sp. in the head kidney samples of dead fish (Fig. 4). This was done as previously described [10], where the values obtained in this challenge were compared to those from a spiked standard curve. Statistical analysis did not detect any significant differences in bacterial load between the two test vaccines and the PBS-vaccinated control.

3.4. In vitro experiments

Confocal microscopy of leucocytes exposed to *Pasteurella* sp. showed that bacteria were possibly present inside the cells both at 6 h and at 24 h post infection (Fig. 5). In SEM images (Fig. 6), bacteria were visible as individual cells on the surface as well as possibly underneath the surface of the leucocytes at 6 h post infection.

This is also seen in the confocal images (Fig. 5), where bacteria are

ARTICLE IN PRESS

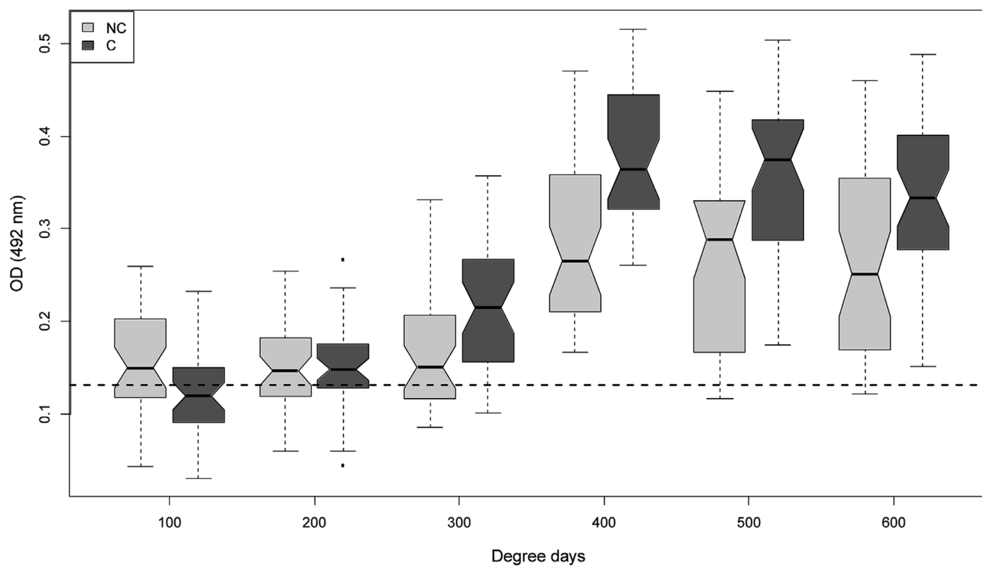

Fig. 1. Antibody levels (OD) post-vaccination measured by ELISA. The horizontal dashed line represents the non-vaccinated baseline control; the boxes represent the first and third quartiles, while the thick solid horizontal line represents the median. The whiskers represent the range of values recorded. (NC: Non-concentrated vaccine, C: Concentrated vaccine).

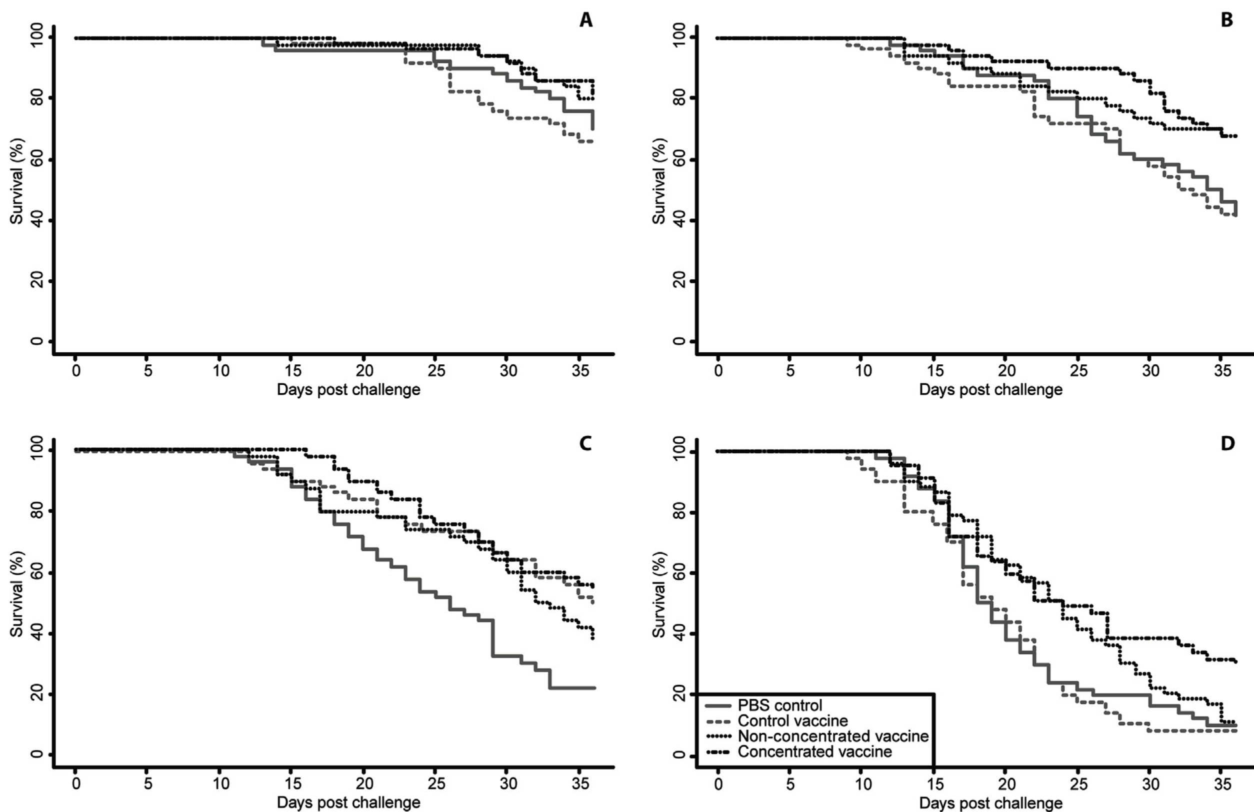

Fig. 2. Survival curves resulting from the vaccine efficacy trial using different bath challenge doses. A: 1×10^5 bacteria mL^{-1}, B: 5×10^5 bacteria mL^{-1}, C: 1×10^6 bacteria mL^{-1}, D: 1×10^7 bacteria mL^{-1}. n = 100 per challenge dose. The results from identical duplicate tanks are combined prior to presentation.

Table 2
Relative percent survival (RPS) at the end of the vaccine trial. *p-value: comparison of mortalities from the two vaccine groups at the end of the trial and set at p = 0.05. The lowest challenge dose is not included in the analysis due to low mortalities observed.

Challenge dose (bacteria mL^{-1})	Concentrated vaccine RPS (%)	Non-concentrated vaccine RPS (%)	p-value*
5×10^5	44.83	44.83	1
1×10^6	41.03	20.51	0.39
1×10^7	21.82	1.27	**0.03**

seen as red dots within the cell, in and around the nucleus of the leucocyte. The bacteria seem to aggregate into chains which gradually become coiled, as can be seen from the SEM and confocal images at 24 h. They also appear to be attached to the surface of the cell. This coiling effect was also observed from Gram stains of the bacteria kept in L-15/FCS and TSB for 24 h, but not in Gram stains done after 6 h of the bacteria in L-15/FCS or TSB (data not shown), and in confocal photomicrographs. As can be noted in Fig. 5B, the bacteria become slightly more elongated and narrower with time as they accumulate to form the coiled chains. The effect of the bacteria on the leucocytes can also be observed with time as these become apoptotic with time but appear

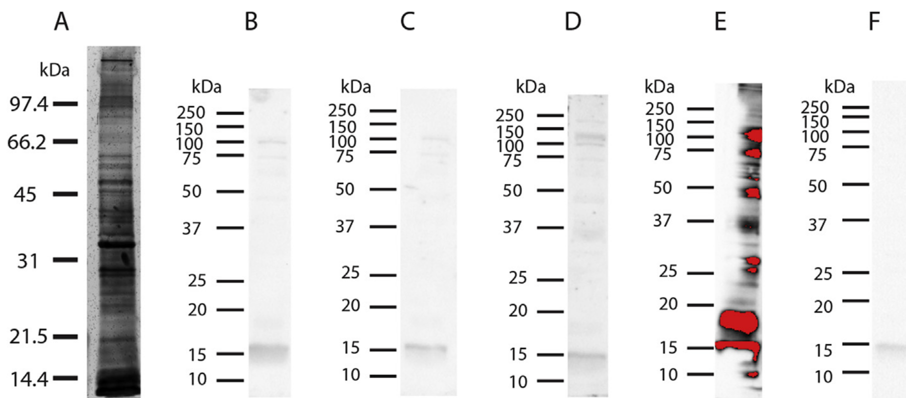

Fig. 3. SDS-PAGE silver stained protein profile of *Pasteurella* sp. bacterial components and serum antibody specificity analysed by western blots. A: silver stained protein profile of *Pasteurella* sp., B: Western blot using serum from fish vaccinated with non-concentrated *Pasteurella* sp. vaccine, C: Western blot using serum from fish vaccinated with concentrated *Pasteurella* sp. vaccine, D: Western blot using serum from fish triple immunised with *Pasteurella* sp. E: Western blot using rabbit anti-*Pasteurella* serum, F: Western blot using serum from non-vaccinated lumpsuckers.

morphologically intact and healthy at 6 h post infection.

4. Discussion

Vaccination of lumpsucker using *Pasteurella* sp. bacterins has, to our knowledge, not been carried out. This has mostly been due to difficulties in culturing the newly encountered pathogen in liquid media. In this work, we used results obtained from previous experiments in order to enhance our understanding of *Pasteurella* sp. and to establish preventative measures against pasteurellosis in lumpsuckers.

ELISA analysis of sera collected from vaccinated fish showed that specific antibodies are generated in relatively high amounts following vaccination with *Pasteurella* sp. bacterins. This is supported by earlier findings showing that *Pasteurella* sp. is highly immunogenic in lumpsucker, providing high levels of specific antibodies after triple immunisation [9]. Moreover, the concentrated vaccine resulted in higher levels of specific antibodies generated, which was likely related to the increased antigen dose. In the concentrated preparation of this vaccine, the bacteria were centrifuged and approximately 90% of the supernatant was discarded prior to further processing. Therefore, the majority of any extracellular proteins (ECPs) produced by the bacteria were not included, unlike the non-concentrated vaccine. However, the number of inactivated bacterial cells per mL were approximately 10-fold higher for the concentrated vaccine, and the slightly higher protection after challenge observed for the concentrated vaccine was likely due to the increased antigen content per dose.

The vaccines did not provide complete protection against the disease. Although the onset of mortality was delayed and the total levels of mortality somewhat reduced, the protection provided by the vaccines was relatively weak. Quite high mortalities still occurred for the vaccinated groups, with approximately 40% relative protection (RPS) at the end of the trial at best. Although the infection pressure in the highest challenge dose may have masked the effect of the vaccine, resulting in the lack of protection observed, this was also seen in the next weaker dose, where mortality in the vaccinated groups was higher than 40%.

The clinical signs of diseased fish observed in this experiment were similar to those reported previously [10]. Of interest was the observation of an additional clinical sign in this study i.e. the frayed fins in the early stages of the infection in the higher two challenge doses used in the trial. This was also observed during a natural outbreak of pasteurellosis in lumpsuckers [3]. Development of these clinical signs together could be due to the different challenge pressure and dynamics present in this trial.

Both experimental vaccines induced significantly increased levels of specific antibodies. A weak trend was detected where the concentrated vaccine consistently provided slightly better protection compared to the non-concentrated vaccine and this might be related to the increased antibody levels. However, significant differences in RPS between the two groups at the end of the challenge could only be proven for the

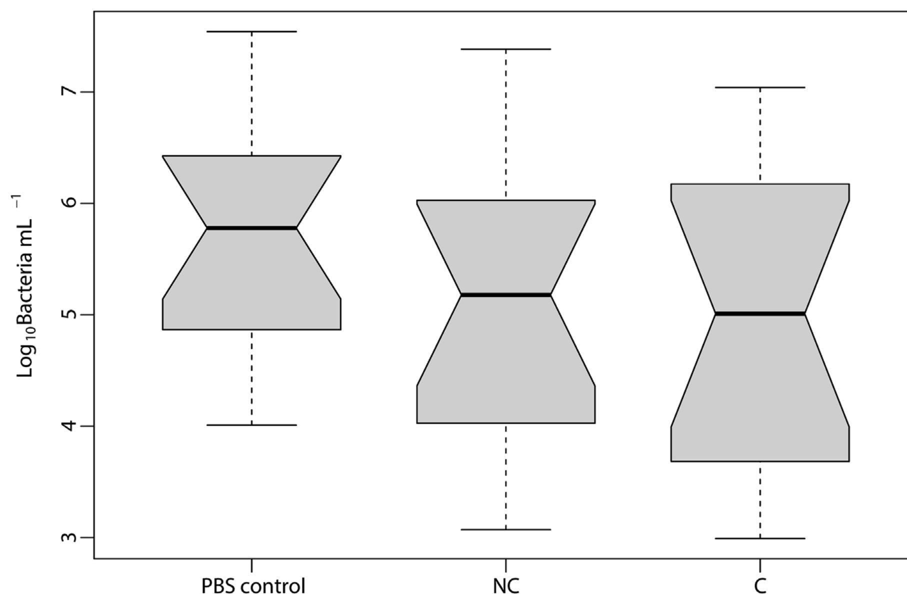

Fig. 4. qPCR confirmation of presence of *Pasteurella* sp. in dead challenged vaccinated and control fish. The boxes represent the first and third quartiles, while the thick solid horizontal line represents the median. The whiskers represent the range of values recorded. (NC: Non-concentrated vaccine, C: Concentrated vaccine).

ARTICLE IN PRESS

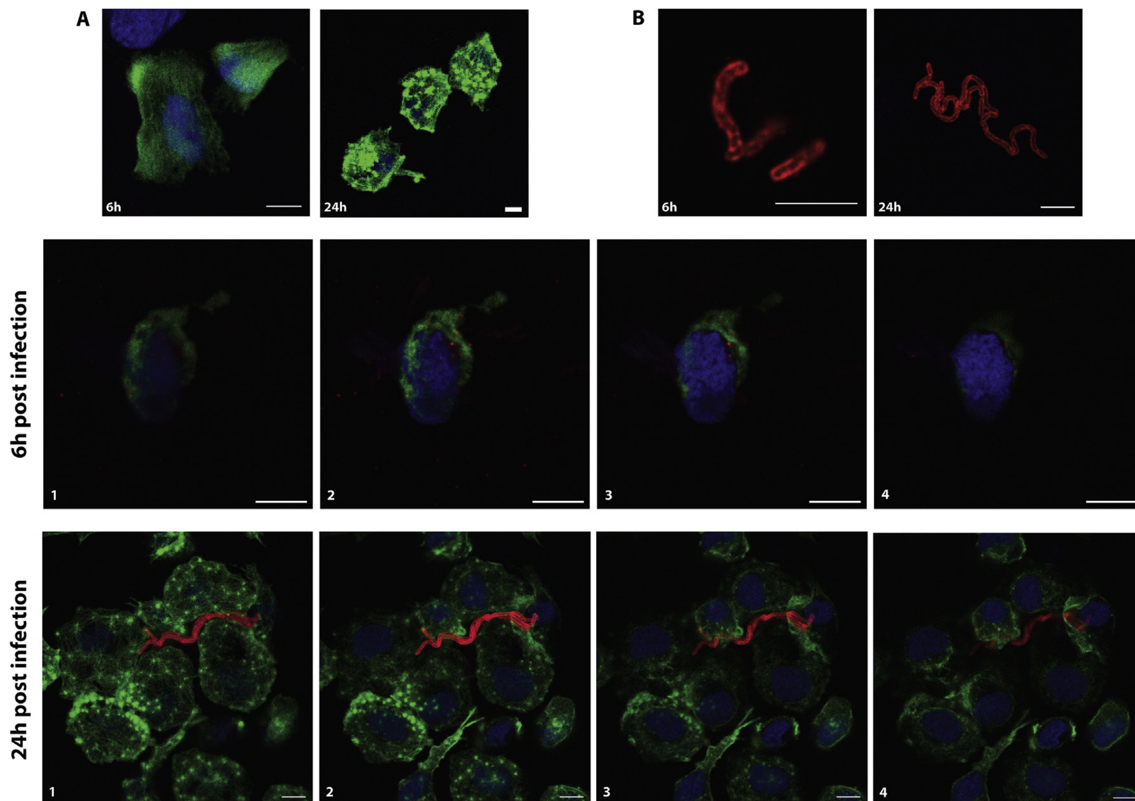

Fig. 5. Confocal micrographs after *in vitro* infection of head kidney leucocytes (HKL) with *Pasteurella* sp. Leucocyte actin filaments and nuclei are stained green and blue, respectively, while bacteria are stained red. A: Non-infected HKL. B: Isolated *Pasteurella* sp. Top series: sections through an infected HKL, 6 h post infection. Bottom series: sections through infected HKL, 24 h post infection. All scale bars: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

highest challenge dose.

Host-pathogen interactions between *Pasteurella* sp. and lumpsucker leucocytes as observed in this study have also been noted for other pathogens that interfere with the immune system of the host. *M. viscosa* eludes protective immune responses of the host by suppressing the production of IL1β, hence delaying bacterial elimination [15], while *Yersinia ruckeri* [16] and *Francisella noatunensis* subsp. *noatunensis* [17–19] reside and replicate within macrophages.

The immunoblot showing only a few highly antibody-reactive bands from the SDS-PAGE separated bacterial components might suggest that the specificity and reactivity of the antibodies is not directed towards major cell wall-integrated components. This might partly explain the limited protection conferred. The reason for the low number of reactive bands on the immunoblot can be that the proteins are glycosylated and not well transferred to the membrane. If any loosely attached carbohydrate antigens are present in the antigen preparations of the vaccines,

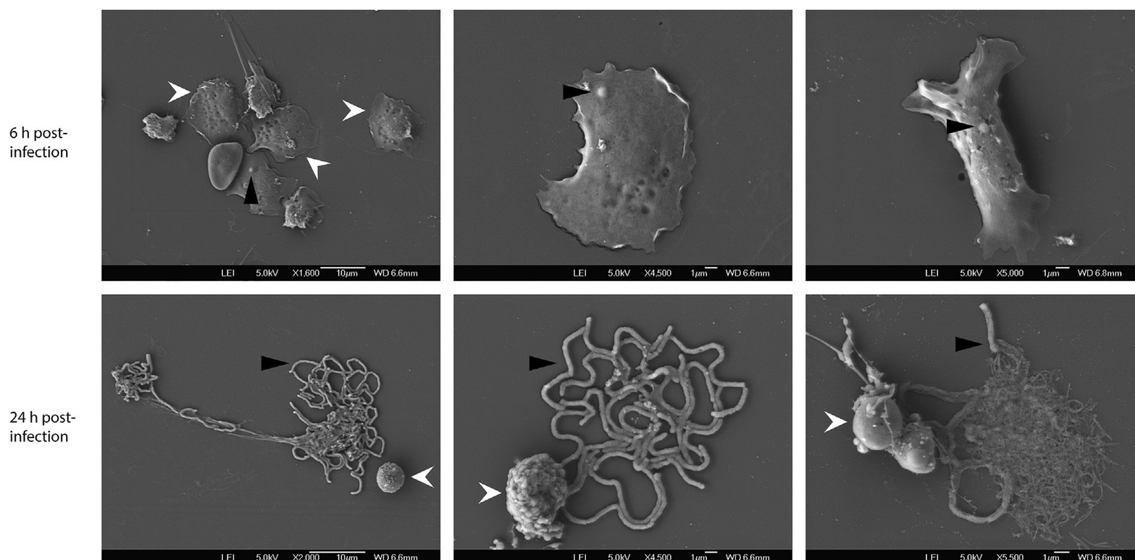

Fig. 6. Scanning electron micrographs (SEM) of head kidney leucocytes at two time points post *in vitro* infection. Scale bar = 10 μm for the first image in each series, 1 μm for the two following images. White arrows indicate leucocytes, while black arrows indicate bacteria.

they can significantly contribute to the high levels of antibodies observed in ELISA.

The presence of carbohydrate and protein reactive fish antibodies to bacterial cell surface components can be detected by other methods [20]. However, there is an obvious difference observed using rabbit serum where many antibody-reactive bands are seen in the immunoblot. Thus, bacterial components are well transferred to the nitrocellulose membrane, and the differences observed between lumpsucker sera and rabbit sera might be species dependent and also dependent on immunisation procedure and adjuvants, since there are more bands from the triple immunised lumpsucker serum, than from the serum from the vaccinated lumpsuckers.

Previous studies on the adaptive immune responses of lumpsucker indicated that IgM^+ B cells abundant in blood were highly phagocytic, and provided a specific antibody response following immunisation [9]. Further work [14] showed that lumpsucker vaccinated with atypical *A. salmonicida* developed a strong humoral immune response to the bacteria, which resulted in significant protection of the fish against bacterial disease tested by challenge. This was also investigated in another study [21], where it was found that specific antibody responses to *V. anguillarum* serotype 01 and *M. viscosa* were lower than towards atypical *A. salmonicida*. The levels of antibodies to the various bacteria recorded in these studies are in the same range as those identified in the current work. However, in contrast to the previously mentioned studies for other bacteria, protection against *Pasteurella* sp. in the present study was relatively weak.

The potency of commercial vaccines must be tested by means of correlates of protection. For fish vaccines, this is usually performed by vaccination-challenge experiments using the pathogens the vaccine is intended to protect against. However, it is also acceptable to use appropriately validates alternative methods not involving challenge of fish. One example of such correlates are specific immune responses to antigens present in a vaccine that elicit protection against an infection or disease [22]. Therefore, vaccines should ideally induce opsonophagocytic and/or neutralising antibodies and should activate immune functions mediated by $CD4^+$ and possibly also $CD8^+$ cells [22]. Moreover, antibody levels measured post-vaccination *in vitro* (such as through ELISA) will not distinguish between neutralising and non-neutralising antibodies, and typically the latter would be antibodies that are not involved in opsonophagocytosis [22] or complement mediated killing. Such antibodies would be surrogates of protection, since they are straightforward to measure, but not directly related to the correlate of protection.

From confocal microscopy of *in vitro* infected leucocytes, one can see aggregated bacteria interacting with the leucocytes. In a previous study [10], diffuse bacterial aggregates were observed in tissue samples from challenged lumpsucker. Whether this take place in natural infections is not clear, but bacterial aggregates would possibly have an impact on the antibody functionality and cause suboptimal bacterial clearance. These factors could possibly explain the results obtained from our vaccine trial with findings of bacterial specific antibodies, but a lack of protection in challenge experiments. Thus, despite the vaccines being capable of eliciting B cell production of specific antibodies, these did not provide sufficient protection from disease and mortality.

The possibility of *Pasteurella* sp. being intracellular, may be indicated from the immune detection of bacteria through confocal microscopy and to an extent, through SEM photomicrographs, as bacteria are possibly found inside leucocytes. Whether this bacterium can reside or even replicate within cells remains to be studied. In that case, improved activation of cellular immune responses could be important to achieve better protection against pasteurellosis. The aggregation of bacteria seen during *in vitro* assays may be a survival strategy to protect against either leucocytes activities, or the incubation medium itself, or other factors including changes in pH. However, considering the results from SEM and confocal microscopy, it cannot be excluded that *Pasteurella* sp. may be a facultative intracellular pathogen and possibly, therefore, stimulation of cellular immunity may be vital to achieve protection of lumpsucker against *Pasteurella* sp.

Agglutination tests carried out showed that the immune sera raised against the bacteria agglutinated live *Pasteurella* sp. indicating that binding to bacterial surface components does occur. It is tempting to speculate, however, considering the low protection obtained by the tested vaccines, that an extracellular capsule, proteins or toxins may be involved in disease progression. If toxic extracellular proteins were involved, extensive genome analysis would be required in order to identify the specific virulence factors and consider if antibodies with antitoxin properties can be obtained.

5. Conclusion

In summary, despite lumpsuckers responding to vaccination with monovalent inactivated *Pasteurella* sp. vaccines by producing specific antibodies, the protection against experimental challenge was relatively weak. A reason for this could potentially be that a specific antibody response is not enough to provide complete protection. This indicates that parts of the immune system other than the humoral part could be important for protection against pasteurellosis, highlighting the need for further work on the mechanism of infection of *Pasteurella* sp. in lumpsucker. Additionally, there might be pathogen specific virulence factors involved in disease development such as intracellular localisation, extracellular and toxic proteins, as well as aggregation. Considering all these results and observations, we think the virulence factors of *Pasteurella* sp. isolate from lumpsuckers should be thoroughly investigated.

Declaration of competing interest

The authors state that there are no competing interests to declare regarding the presented work.

Acknowledgements

The authors would like to thank Mr Paul Løvik for his technical assistance during this work.

These trials were approved by the Norwegian Food Safety Authority, National Assignments Department, under approval numbers 17605. This work was supported by the University of Bergen and integrated with Norwegian research Council project no. 244148: The Cleaner fish Lumpfish (Cyclopterus lumpus L.) - Immunity, Diseases and Health). Vaccination of the fish was financed by PHARMAQ part of Zoetis. The confocal imaging and SEM of leucocytes was performed at the Molecular Imaging Centre (FUGE, Norwegian Research Council), University of Bergen.

References

[1] C. Walde, S. Gulla, H. Hansen, E. Mjølenrød, G. Bornø, The health situation of cleaner fish, in: B. Hjeltnes, B. Bang Jensen, G. Bornø, A. Haukaas, C. Walde (Eds.), Fish Heal. Rep. 2018, Norwegian Veterinary Institute, Oslo, Norway, 2019, pp. 119–125.

[2] M. Marcos-López, K. Donald, H. Stagg, U. McCarthy, Clinical *Vibrio anguillarum* infection in lumpsucker *Cyclopterus lumpus* in Scotland, Vet. Rec. 173 (2013) 319, https://doi.org/10.1136/vr.101763.

[3] M. Alarcón, S. Gulla, M.V. Røsæg, A. Rønneseth, H. Wergeland, T.T. Poppe, H. Nilsen, D.J. Colquhoun, Pasteurellosis in lumpsucker *Cyclopterus lumpus*, farmed in Norway, J. Fish Dis. 39 (2016) 489–495, https://doi.org/10.1111/jfd.12366.

[4] G. Bornø, M. Alarcón, M. Linaker, D. Colquhoun, H. Nilsen, J. Gu, B. Gjerset, H. Hansen, E. Thoen, S. Gulla, B. Bang Jensen, Akutt Dødelighet Hos Rognkjeks (*Cyclopterus lumpus*) I 2015, (2016) Oslo, Norway.

[5] G. Bornø, S. Gulla, The health situation of cleaner fish, in: B. Hjeltnes, C. Walde, B. Bang-Jensen, A. Haukaas (Eds.), Fish Heal. Rep. 2015, Norwegian Veterinary Institute, Oslo, Norway, 2016, pp. 70–73.

[6] T.H. Birkbeck, L.A. Laidler, A.N. Grant, D.I. Cox, *Pasteurella skyensis* sp. nov., isolated from Atlantic salmon (*Salmo salar* L.), Int. J. Syst. Evol. Microbiol. 52 (2002) 699–704, https://doi.org/10.1099/ijs.0.01884-0.

[7] M. Valheim, T. Håstein, E. Myhr, L. Speilberg, H.W. Ferguson, Varracalbmi: a new

bacterial panophthalmitis in farmed Atlantic salmon, *Salmo salar* L. J. Fish Dis. 23 (2000) 61–70, https://doi.org/10.1046/j.1365-2761.2000.00209.x.

[8] G.T. Haugland, R.A. Jakobsen, N. Vestvik, K. Ulven, L. Stokka, H.I. Wergeland, Phagocytosis and respiratory burst activity in lumpsucker (*Cyclopterus lumpus* L.) leucocytes analysed by flow cytometry, PLoS One 7 (2012), https://doi.org/10.1371/journal.pone.0047909.

[9] A. Rønneseth, D.B. Ghebretnsae, H.I. Wergeland, G.T. Haugland, Functional characterization of IgM+ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus* L.), Dev. Comp. Immunol. 52 (2015) 132–143, https://doi.org/10.1016/j.dci.2015.05.010.

[10] R.M. Ellul, C. Walde, G.T. Haugland, H. Wergeland, A. Rønneseth, Pathogenicity of *Pasteurella* sp. in lumpsuckers (*Cyclopterus lumpus* L.), J. Fish Dis. 42 (2019) 35–46, https://doi.org/10.1111/jfd.12905.

[11] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685, https://doi.org/10.1038/227680a0.

[12] V.C.W. Tsang, J.M. Peralta, A. Ray Simons, Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis, Methods Enzymol. 92 (1983) 377–391, https://doi.org/10.1016/0076-6879(83)92032-3.

[13] R.C. Switzer, C.R. Merril, S. Shifrin, A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels, Anal. Biochem. 98 (1979) 231–237, https://doi.org/10.1016/0003-2697(79)90732-2.

[14] A. Rønneseth, G.T. Haugland, D.J. Colquhoun, H.I. Wergeland, E. Brudal, Protection and antibody reactivity following vaccination of lumpfish (*Cyclopterus lumpus* L.) against atypical *Aeromonas salmonicida*, Fish Shellfish Immunol. 64 (2017) 383–391, https://doi.org/10.1016/j.fsi.2017.03.040.

[15] M. Løvoll, C.R. Wiik-Nielsen, H.S. Tunsjø, D. Colquhoun, T. Lunder, H. Sørum, S. Grove, Atlantic salmon bath challenged with *Moritella viscosa* - pathogen invasion and host response, Fish Shellfish Immunol. 26 (2009) 877–884, https://doi.org/10.1016/j.fsi.2009.03.019.

[16] J. Ryckaert, P. Bossier, K. D'Herde, A. Diez-Fraile, P. Sorgeloos, F. Haesebrouck, F. Pasmans, Persistence of *Yersinia ruckeri* in trout macrophages, Fish Shellfish Immunol. 29 (2010) 648–655, https://doi.org/10.1016/j.fsi.2010.06.009.

[17] A. Furevik, E.F. Pettersen, D. Colquhoun, H.I. Wergeland, The intracellular lifestyle of *Francisella noatunensis* in Atlantic cod (*Gadus morhua* L.) leucocytes, Fish Shellfish Immunol. 30 (2011) 488–494, https://doi.org/10.1016/j.fsi.2010.11.019.

[18] K.R. Bakkemo, H. Mikkelsen, M. Bordevik, J. Torgersen, H.C. Winther-Larsen, C. Vanberg, R. Olsen, L.H. Johansen, M. Seppola, Intracellular localisation and innate immune responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages, Fish Shellfish Immunol. 31 (2011) 993–1004, https://doi.org/10.1016/j.fsi.2011.08.020.

[19] N. Vestvik, A. Rønneseth, C.A.K. Kalgraff, H.C. Winther-Larsen, H.I. Wergeland, G.T. Haugland, *Francisella noatunensis* subsp. *noatunensis* replicates within Atlantic cod (*Gadus morhua* L.) leucocytes and inhibits respiratory burst activity, Fish Shellfish Immunol. 35 (2013) 725–733, https://doi.org/10.1016/j.fsi.2013.06.002.

[20] P.A. Wood, S.L. Kaattari, Enhanced immunogenicity of *Renibacterium salmoninarum* in chinook salmon after removal of the bacterial cell surface-associated 57 kDa protein, Dis. Aquat. Org. 25 (1996) 71–79, https://doi.org/10.3354/dao025071.

[21] T. Erkinharju, M.R. Lundberg, E. Isdal, I. Hordvik, R.A. Dalmo, T. Seternes, Studies on the antibody response and side effects after intramuscular and intraperitoneal injection of Atlantic lumpfish (*Cyclopterus lumpus* L.) with different oil-based vaccines, J. Fish Dis. 40 (2017) 1805–1813, https://doi.org/10.1111/jfd.12649.

[22] S.A. Plotkin, Vaccines: correlates of vaccine-induced immunity, Clin. Infect. Dis. 47 (2008) 401–409, https://doi.org/10.1086/589862.