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Antigenic variability of *Vibrio anguillarum* serotype O2a: a hurdle for vaccine efficacy against vibriosis in *Oncorhynchus mykiss*

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Abstract

Despite vaccination, outbreaks of vibriosis still occur in sea-reared rainbow trout in Denmark. Vibriosis outbreaks are caused mainly by *V. anguillarum* serotypes O1 and O2a, and bacterins of both serotypes are included in the commonly used vaccine against this disease in Danish aquaculture. However, while the strains belonging to serotype O1 are genetically similar, the strains belonging to serotype O2a are highly diverse. This work aimed first at examining how the antibody response and protection induced by bacterin-based vaccines were affected by the antigenic variability within *V. anguillarum* serotype O2a strains. Following vaccination of rainbow trout with either a commercial or an experimental vaccine, specific antibody reactivity in serum from vaccinated fish was examined by ELISA against 23 strains of *V. anguillarum* serotype O2a (VaO2a). The strains were divided into 4 distinct subgroups according to the observed detection pattern. Seven strains were strongly recognized only by sera from fish vaccinated with the experimental vaccine (EV-I antisera), while 13 other strains were primarily recognized by sera from fish vaccinated with the commercial vaccine (CV antisera). Two strains were recognized by both EV-I and CV antisera, but with intermediate reactivity, while one strain was not recognized at all. A partly similar recognition pattern was observed when purified lipopolysaccharide (LPS) was used as antigen in the examination of antibody reactivity in Western blotting. The level of protection was highly dependent on both the vaccine and the strain used for challenge and showed no consistent correlation with antibody reactivity. Secondly, we attempted to use a bacterin vaccine based on one of the *V. anguillarum* O2a strains intermediately recognized by both EV-I and CV antisera to investigate whether that could potentially provide protection across strain variability. The immunized fish did mount a cross-reactive antibody response, but protection still varied depending on the strain used for challenge.

Interestingly, the grouping of strains according to antibody reactivity correlated not only with genotyping based on single nucleotides polymorphisms analysis (SNP) but also with variability in the accessory genome, indicating that presence or absence of protein antigens or proteins associated with the biosynthesis of antigenic epitopes may explain the observed distinct serological subgrouping within VaO2a strains by trout immune sera.

In terms of vaccination against VaO2a, our results demonstrate that it is important to take (local) antigen variations into account when using bacterin-based vaccines but also that alternatives to traditional bacterin-

based vaccines might be needed to induce protection against the highly virulent *Vibrio anguillarum* serotype O2a strains.

1. Introduction

Vibriosis, fatal haemorrhagic septicaemia, caused by the Gram-negative bacterium *Vibrio anguillarum*, has long been one of the most prevalent bacterial diseases in sea-reared salmonids [1–3]. Vaccines against vibriosis have played an important role in preventing this disease in farmed fish, and along with this, improving animal welfare, decreasing economic losses, and reducing the amount of antibiotics used for treatment [4–6]. It is a common practice for the Danish marine aquaculture industry to vaccinate rainbow trout against vibriosis and furunculosis before moving the fish into sea cages. The vaccine formulations are based on inactivated whole-cell bacterins, including *Aeromonas salmonicida* and *V. anguillarum* serotypes O1 and O2a (VaO1 and (VaO2a, respectively), both serotypes associated with vibriosis outbreaks in salmonids [7,8]. Despite vaccination, disease outbreaks of furunculosis and occasionally vibriosis still occur [2]. Based on the comparison of sequences of 44 strains of *V. anguillarum* isolated mostly from vibriosis outbreaks in rainbow trout in Denmark, the strains belonging to serotype O1 are genetically similar. In contrast, strains belonging to serotype O2a are genetically diverse [9]. We have recently observed strain-dependent variability in vaccine-induced protection against VaO2a when comparing a commercial and an experimental bacterin based vaccine [10]. Similar observations have been done in studies on vibriosis vaccines in Atlantic cod (*Gadus morhua*), where the antigenic variability of *V. anguillarum* serotype O2 (VaO2) resulted in lower protection against vibriosis caused by a VaO2 strain heterologous to the strain used in the vaccine formulation. Broader protection was obtained only when the vaccine formulation included strains of several VaO2 subgroups (O2a, O2b, and an atypical O2) [11,12], suggesting that strain-related antigen variability should be taken into account when designing effective vaccines against vibriosis caused by highly variable serotypes like VaO2. This variability among the *Vibrio anguillarum* strains belonging to the same serotype indicates that classical serotyping based on rabbit immune sera raised against the lipopolysaccharides (LPS) component of the cell wall of Gram-negative bacteria and specifically the heat-stable outer polysaccharide region of the LPS on the O-antigen is insufficient as a tool for selecting vaccine antigens [7,13]. Early studies accordingly suggested that fish antibodies may recognize other sections of LPS molecules or other variable non-LPS VaO2 antigens. [13,14].

This study aimed first to elucidate how the antibody response and protective efficacy of an experimental and a commercial vaccine in rainbow trout were affected by the genetic variability of VaO2a. Secondly, we aimed to test whether a bacterin vaccine based on a serologically broadly recognized single strain of VaO2a could potentially induce protection against vibriosis across the genetic variability of VaO2a strains.

2. Material and methods

2.1. Experimental fish

Outbred all-female rainbow trout-eyed eggs were obtained from disease-free farms, surface disinfected by iodine treatment before introduction into pathogen-free experimental facilities, where they were hatched and grown until used for vaccination trials. All experiments were approved by the Animal Experiments Inspectorate, Ministry of Environment and Food under the license 2014-15-0201-00098.

2.2. Bacterial strains

Twenty-three *Vibrio anguillarum* serotype O2a strains (Table 1) were used in this study. Nineteen strains were isolated from vibriosis outbreaks in Danish rainbow trout farms between 1976 and 2017, as previously described [9,15]. Four strains were isolated from other fish species; among these, two were from European flounder (*Platichthys flesus*), one from cod (*Gadus morhua*), and one from Northern pike (*Esox lucius*). The latter was isolated in Finland, while all other strains were from Denmark. The genome sequences of the 23 *V. anguillarum* strains are available in GenBank [9].

2.3. Vaccines

All vaccines were based on whole-cell bacterin antigen prepared by formalin-inactivation of bacterial cells. For trial I, an experimental pentavalent vaccine and a trivalent commercial vaccine earlier described in Marana et al.[10] were applied. The experimental and commercial vaccines were renamed for this work as EV-I and CV, respectively. The experimental vaccine (EV-I) was formulated with mineral oil adjuvant and cells from five bacterial strains belonging to: *Yersinia ruckeri* serotype O1 biotype 1 and 2, *Vibrio anguillarum* serotype O1 and O2a, and *Aeromonas salmonicida* subsp. *salmonicida*. All these strains were isolated from Danish rainbow trout farms [10]. For VaO2a, the strain used in the EV-I formulation was 090903-1/2B. The commercial vaccine (CV) was formulated with mineral oil adjuvant and cells of three bacterial strains representing: *V. anguillarum* serotype O1 and O2a, and *A. salmonicida* subsp. s*almonicida*. (Alphaject 3000, Pharmaq, Norway). The strains used for CV formulation are not disclosed by the supplier. Saline was used as negative control. For trial II, the experimental vaccine II (EV-II) was formulated with *V. anguillarum* serotype O2a strain 090819-1/29 bacterin. This strain was grown in Veal infusion medium (Difco) for 48 h at 20 °C. When the bacterial culture reached an OD_{600} of 1.5, corresponding to 5.5×10^9 cfu/ml, the cells were centrifuged at 2500 × g and resuspended in an equal volume of PBS. Inactivation was performed by adding formaldehyde to a final concentration of 0.7% followed by overnight (o.n.) incubation at 4 °C. Following centrifugation and resuspension in PBS, sterility was confirmed by plating on blood agar plates. This bacterin was emulsified with an equal volume of Freund´s Incomplete Adjuvant (FIA) before being used for vaccination. PBS was used as negative control.

2.4. Vaccination and challenge- Trial I

The fish were hatched and vaccinated in a pathogen-free facility in Bornholm Salmon Hatchery (Nexø, Denmark) as described earlier [10]. In short, fish at an average size of 34 g were anaesthetized with Finquel®Vet (100 mg/l) and vaccinated by injecting intraperitoneally (IP) 0.1 ml of either: the commercial vaccine (CV), the experimental vaccine (EV-I), or 0.9% NaCl (Saline). Fish were tagged by removing either: the upper part of the left maxilla (EV-I), the adipose fin (CV), or the upper part of the right maxilla (Saline). The groups were mixed, and fish were kept in 700-l aerated tanks with fresh water at 12 °C. One week before the infection trial, the fish were transported to the fish facility at the Technical University of Denmark (DTU), Kongens Lyngby, Denmark.

At 57 days post-vaccination (dpv) (684 degree-days), the fish (85-120 g) were divided into seven 180-l aerated tanks. Each tank contained 48 fish in total, 16 fish from each treatment: EV-I, CV, and Saline. After anaesthetizing the fish by immersion in water with benzocaine (0.01%), the challenge was performed by IP injection of 0.1 ml of either: Veal infusion medium as a negative control or diluted o.n. cultures of *V. anguillarum* O2a strains: 090903-1/2B (1×10^8 cfu/ml), 090819-1/29A (1×10^7 cfu/ml), or 090707-1/2A (1×10^7 cfu/ml). The fish injected with the Veal infusion medium were kept in one tank, while the fish injected with each *V. anguillarum* strain were kept in duplicated tanks. The virulence of these isolates was different, and the dose of bacteria used for the challenge was adjusted according to a preliminary challenge experiment (data not shown) aiming at 60-90% of the fish developing clinical signs.

The morbidity of fish was monitored for 12 days post-challenge. Fish showing clinical signs of disease (abnormal swimming behaviour) were euthanized with an overdose of benzocaine and registered as mortality. Re-isolation of the bacteria was performed from a representative number of fish from each tank by taking swabs from the head kidney and plating them on blood agar plates, followed by serological identification [16].

2.5. Vaccination and challenge- Trial II

The fish were hatched and reared in an enclosed pathogen-free facility at DTU. After anaesthetizing fish (60-100 g) by immersion in water with benzocaine (0.01%), the vaccination was performed by two IP injections of 50 µl each of either EV-II or PBS (120 fish in each group). The fish were tagged subcutaneously with Visible Implant Elastomer (VIE) Tags (Northwest Marine Technology) in the lower right jaw (EV-II) or the lower left jaw (PBS). The groups were mixed and kept in two aerated 180-l tanks with fresh water at 12 °C until the bacterial challenge.

At 51 dpv (612 degree-days), the fish (80-150g) were divided into seven aerated 180-l tanks. Each tank contained 35-36 fish in total, 17-18 fish of each group: EV-II and PBS. After anaesthetizing the fish with 0.01% benzocaine, the challenge was performed by IP injection of 0.1 ml with either: Veal infusion medium

as negative control, *V. anguillarum* serotype O2a strains: 090903-1/2B (1.0×10^9 cfu/ml), 090819-1/29A (2.2×10^7 cfu/ml), or 090707-1/2A (5.4×10^7 cfu/ml). The fish injected with the Veal infusion medium were kept in one tank, while the fish injected with each *V. anguillarum* strain were kept in duplicated tanks. The morbidity of fish was monitored during 28 days post-challenge. Registration of disease, termination, and re-isolation of the bacteria was performed as described in trial I.

2.6. Serum sampling

For each experimental group, blood samples from unchallenged fish were taken at 86 dpv in trial I and at 70 dpv in trial II. For trial I, ten fish vaccinated with EV-I, nine fish vaccinated with CV, and 3 fish injected with Saline were sampled. For trial II, ten fish vaccinated with EV-II and 3 fish injected with PBS were sampled. Fish were anaesthetized by immersion in water with benzocaine (0.01%), and blood was collected from the caudal vein in 2 ml tubes. After clotting at 4°C o.n., blood samples were centrifuged at 4000 × g for 15 min at 4°C and sera were collected and stored at -80°C until analysis.

2.7. Examination of antibody reactivity by ELISA

Twenty-three strains of *V. anguillarum* serotype O2a (Table 1) were grown in 5 ml of Veal infusion medium for 48 h at 20°C. The cultures were centrifuged at 3000 × g for 20 min at 4°C. The supernatants were discharged, and the pellets were resuspended in 5 ml of PBS. The resuspended cells were once again centrifuged at 3000 × g for 20 min at 4°C, and the supernatants discharged. Pellets were then resuspended in 2 ml of PBS and sonicated on ice with 10 cycles of 30 s ON, 45 s OFF, at 100 % power (Soniprep 150, MSE). The protein concentration of the sonicated bacteria was quantified by BCA kit (Pierce) according to the manufacturer's protocol using BSA as reference. ELISA plate wells (Greiner Microlon High Binding) were coated with 250 ng of protein diluted in 50 µl of coating carbonate buffer 0.1 M (pH 9.6), or coating buffer without antigen. Following overnight incubation at 4°C, the plates were washed 3 times in PBS-T (PBS-0.05%Tween20) and sequentially incubated with: fish serum samples diluted 1/100 in PBS-T-skim milk (PBS, 0.05%Tween20, 5% skim milk) overnight at 4°C, monoclonal antibody 4C10 anti-trout IgM diluted 1/50 in PBS-T-BSA (PBS, 0.05%Tween20, 1% BSA) for 1h at room temperature [17], and peroxidase-conjugated rabbit anti-mouse immunoglobulins (P0260, DAKO) diluted 1/1000 in PBS-T-BSA for 1h at room temperature. Between incubations, the plates were washed 3 times with washing buffer (PBS-T). Finally, 50 µl of peroxidase substrate, TMB plus (Kem-En-Tec Nordic A/S), was added to each well and incubated at room temperature for 23 min. in the dark. The reaction was stopped by adding 50 µl of sulfuric acid (0.5 N). The plates were read at 450 nm and at 650 nm (for optical background) in an ELISA reader (Synergy HT, Biotek) using the Bio Tek Gen5 software (Agilent). All sera were tested in wells with and without antigen, and the readout values were obtained by subtracting A450-A650 values from wells without antigen from A450-A650 values from wells with antigen (average of two replicates) for each serum sample.

For titration of specific antibody reactivity in sera from trial II, only antigens from VaO2a strains 090903-1/2B, 090819-1/29A, and 090707-1/2A were used to coat the plates. Ten serum samples were 2-fold diluted from 1/800 until 1/409600, and the resulting absorbance values (in the range of 0.2-1.5) were used to generate a linear regression plot for each sample. The intercept and slope of each regression line were used to estimate the serum dilution giving an absorbance 1.0 for the given sample. The titer was defined as the log10 value of the reciprocal value of that serum dilution.

2.8. Lipopolysaccharide purification

Vibrio anguillarum strains were grown in 50 ml of Veil infusion medium for 48 h at 20°C. Twenty-five ml of each culture at OD_{600} = 1.0 was centrifuged at 3000 × g for 20 min at room temperature. The supernatant was discharged, and the pellet was resuspended in 1 ml of PBS and vortexed until no aggregations of bacteria were observed. The resuspended bacteria were incubated at 60°C for 40 min and then centrifuged at 14000 × g for 10 min at room temperature [18]. Four hundred µl of supernatant from each strain were collected and mixed with 0.8 ml of cold 0.375 M magnesium chloride in 95% ethanol. After incubation at –20 °C for 30 min, the samples were centrifuged at 16000 × g for 10 min at 4 °C [19]. Each pellet was resuspended in 130 µl of PBS and mixed with 50µl of 4X Loading buffer (Expedeon) and 20µl of 10X DTT (Expedeon). The samples were incubated at 70°C for 15 min. Then, 5 µl of Proteinase K (25 mg/ml) was added to each sample followed by incubation at 60°C for 3h. These samples were used for SDS-PAGE and Western blot. For this analysis, five representative strains across the genetic variability of VaO2a were selected. These strains belong to distant clusters according to the phylogenetic tree in Fig.4 to have a broader knowledge of VaO2a LPS profiles; two EV-I strains (090903-1/2B and 100719-1/3A), two CV strain 090707-1/2A and 850617-1/1, and one EV-I/CV strain 090819-1/29A. The purified LPS were not quantified, but the starting culture material for LPS preparation was the same for all strains.

2.9. SDS-PAGE and Western blot

For the SDS-PAGE, 15µl of Proteinase K-treated LPS samples were used directly loaded on precast 12% TEO-Tricine gel and run it at 200V for 1h using RunBlue TEO-Tricine buffer (Expedeon) for LPS. For Western blot, LPS samples run in SDS-PAGE were first transferred to a PVDF membrane in a semi-dry electroblotter at 20V, 180 mAmp for 2h (Trans-blot SD, Biorad). After transfer, the membrane was blocked in PBS-T-skim milk for 1h at room temperature. The membranes were sequentially incubated with: a pool of 4 fish sera from the different experimental groups (injected with either EV-I, CV, EV-II, or Saline) diluted 1/100 in PBS-T-skim milk overnight at 4°C, monoclonal antibody 4C10 diluted 1/50 in PBS-T-1%BSA for 1h at room

temperature, and rabbit anti-mouse immunoglobulin (P0260, DAKO) diluted 1/1000 in PBS-T-1%BSA for 1h at room temperature. Initial testing revealed no difference in strain-specific reactivity between the individual serum samples included in the pools (data not shown). Between each antibody layer, the membranes were washed three times with PBS-T for 5 min. Finally, the membranes were incubated with ECL Western Blotting peroxidase substrate (Pierce) according to the manufacturer´s recommendation. The membranes were visualized with chemiluminescence reader GeneGnome(Syngene) and documented using the software GeneSnap version 7.09.17.

2.10. Single nucleotide polymorphisms (SNPs) analysis

SNPs in the full genome sequences of the 23 VaO2a isolates were identified with the pipeline CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) [20] using raw adapter and quality-trimmed reads and the complete genome sequence of the *V. anguillarum* serotype O2 strain VIB12, isolated from sea bream in Greece in 1991, GenBank accession number GCA_002310335.1, as a reference [21]. The analysis was run with default settings, and all indels were excluded. iTOL v4 [22] was used to visualize the phylogenetic tree, including the metadata.

2.11. Pan-genome analysis

The pan-genome was calculated with Roary version 3.12.0 [23], a high speed stand-alone pan-genome pipeline, using GFF3 files produced by Prokka 1.12-beta [24]. The program was run using the default settings, which uses BLASTp for all-against-all comparison with a percentage sequence identity of 95%. Using the output of Roary, the hierarchical gene presence/absence tree created was visualized with Phadango [25].
The Venn diagram was constructed to visualize the associations between all genes in the accessory genome arranged into the four subtyping groups according to the ELISA-specific antibody reactivity (CV strains, EV-I strains, EV-I/CV strains, and no reactivity strain), using the output file from Roary [26]. The Venn diagram was done in R (v.4.1.1) with the package ggvenn (v.0.1.9) [27]

2.12. Statistic analysis

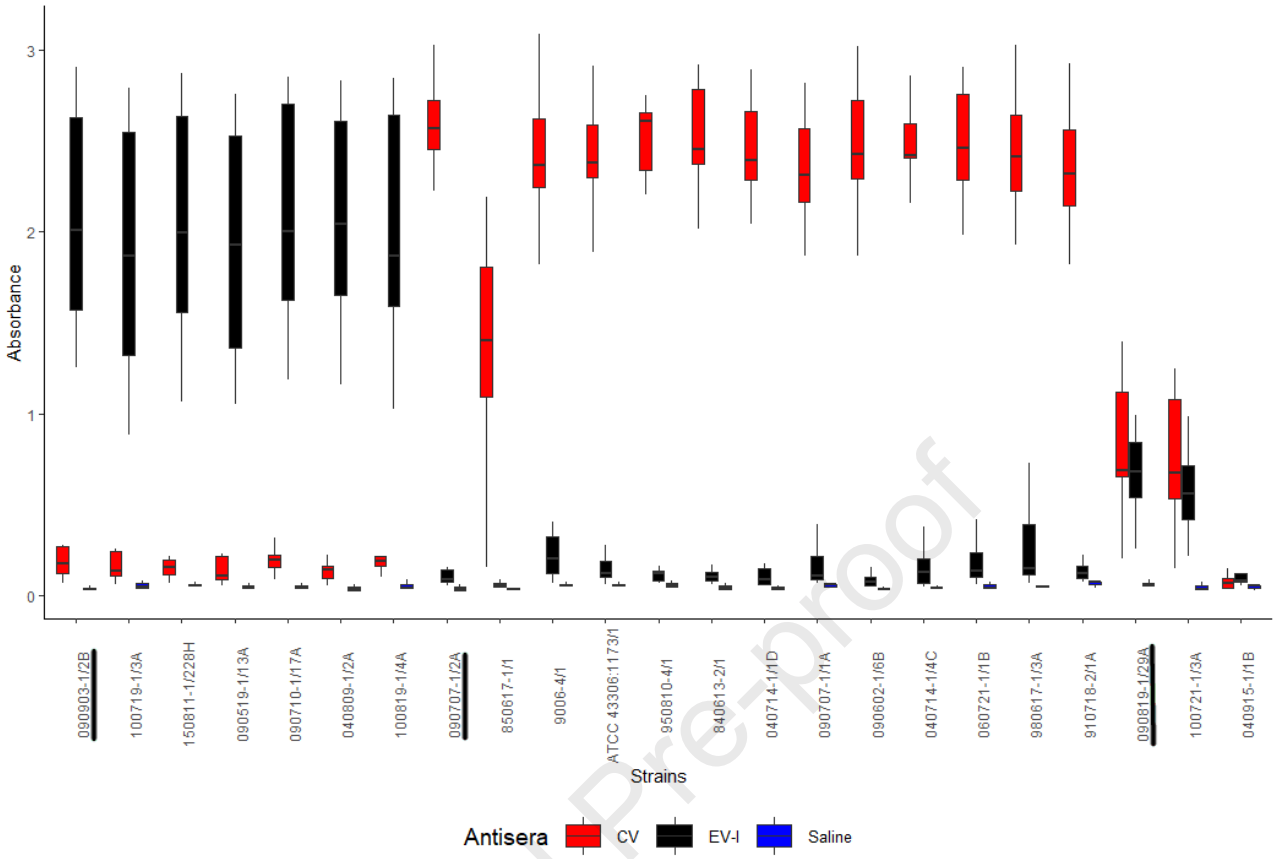
All statistical analyses were done using R (v.4.1.1) [28]. The plots were generated using the R package ggplot2 (v. 3.3.5) [29]. For the ELISA in trial I, the statistical significance of the difference between the reactivities of the EV-I, CV, and saline antisera with each VaO2a strain was determined by ANOVA and using Tukey´s multiple comparison test as post hoc analysis. The same analysis was used to determine the statistical significance of the differences between the titre of strain-specific antibodies in the sera from vaccinated fish against the challenge strains in trial II. The survival analysis and Kaplan-Meier survival curves were generated using R packages “survival” (v.3.2-13) and “survminer” (v.0.4.9) [30,31]. Logistic regression assuming a binomial distribution (binomial GLMM) was applied for comparison of the effect of the different vaccines on

the probability of fish surviving until the end of the experiment. The R package "glmmTMB" (v.1.12.3) was used for this purpose [32]. The multiple comparisons of the endpoint survival between different vaccination groups were made using the R package "emmeans" (v.1.7.1-1) [33]. A detailed explanation of the model and the odd ration calculations were previously published in Barsøe et al. (2021) [34].

3. Results

3.1. Antibody reactivity in sera from fish vaccinated with EV-I and CV (Trial I)

The specific antibody reactivity against VaO2a was examined for ten randomly selected sera from rainbow trout vaccinated with the EV-I (EV-I antisera), nine sera from rainbow trout vaccinated with the CV (CV antisera), and three sera from rainbow trout injected with Saline (Saline antisera). All sera were tested against 23 strains of VaO2a (Table 1). Seven VaO2a strains were strongly recognized by the 10 EV-I antisera (Fig. 1, black bars), while 13 other strains were strongly recognized by the CV antisera (Fig. 1, red bars). Two strains were recognized by EV-I antisera as well as by CV antisera, but the reactivity level was intermediate (090819-1/29A and 100721-1/3A,). Finally, one strain (040915-1/1B) was not recognized by any sera. Saline antisera did not react with any strains (blue bars). These results are also summarised in Table 1. In the following, the strains recognized by EV-I antisera or by CV antisera will be named EV-I strains and CV strains, respectively. The strains recognized by EV-I as well as CV antisera will be named EV-I/CV strains. Finally, the strain that was not recognized by any sera will be named the NR strain.



247

248 **Figure 1. VaO2a strain-specific antibody reactivity** in ELISA of sera from rainbow trout immunized with
249 EV-I (black bars, n = 10), CV (red bars, n = 9), and Saline (blue bars, n = 3). All sera were diluted 1/100 and
250 tested against 23 strains of *V. anguillarum* serotype O2a (annotated on the x-axis). For each EV-I strain, the
251 reactivity with EV-I antisera was significantly different from that of CV antisera and the Saline antisera (p-
252 value < 0.001). Likewise, for each CV strain, the reactivity with CV antisera was significantly different from
253 that of the EV-I antisera and the Saline antisera (p-value < 0.001). The 090903-1/2B strain (first strain on the
254 left) was homologous to the VaO2a bacterin in the EV-I vaccine. Sera from fish injected with Saline displayed
255 background level reactivity against all bacterial strains.

256

257 **Table 1: Origin and serological (ELISA) sub-grouping of *V. anguillarum* O2a strains used in this study**

No.	Strain	Place of isolation	Year of isolation	Host	ELISA group according to reactivity with fish immune sera (see Fig.1)
1	090903-1/2B*	Grønsund (Denmark)	2009	Rainbow trout	EV-I
2	100719-1/3A	Grønsund (Denmark)	2010	Rainbow trout	EV-I

3	150811-1/228H	Grønsund (Denmark)	2015	Rainbow trout	EV-I
4	090519-1/13A	Musholm (Denmark)	2009	Rainbow trout	EV-I
5	090710-1/17A	Grønsund (Denmark)	2009	Rainbow trout	EV-I
6	040809-1/2A	Hjarnø Havbrug (Denmark)	2004	Rainbow trout	EV-I
7	100819-1/4A	Musholm (Denmark)	2010	Rainbow trout	EV-I
8	090707-1/2A	Musholm (Denmark)	2009	Rainbow trout	CV
9	850617-1/1	(Finland)	1985	Northern pike	CV
10	9006-4/1	Skærbæk (Denmark)	1980	Rainbow trout	CV
11	ATCC 43306; 1173/1	(Denmark)	1976	Cod	CV
12	950810-4/1	Snaptun (Denmark)	1995	Rainbow trout	CV
13	840613-2/1	Hjarnø Havbrug (Denmark)	1984	Rainbow trout	CV
14	040714-1/1D	Vejle Fjord (Denmark)	2004	European flounder	CV
15	090707-1/1A	Musholm (Denmark)	2009	Rainbow trout	CV
16	090602-1/6B	Musholm (Denmark)	2009	Rainbow trout	CV
17	040714-1/4C	Vejle Fjord (Denmark)	2004	European flounder	CV
18	060721-1/1B	Hvidesande Dambrug (Denmark)	2006	Rainbow trout	CV
19	980617-1/3A	Musholm (Denmark)	1998	Rainbow trout	CV
20	910718-2/1A	Agersø Havbrug (Denmark)	1991	Rainbow trout	CV
21	090819-1/29A**	Grønsund (Denmark)	2009	Rainbow trout	EV-I/CV
22	100721-1/3A	Grønsund (Denmark)	2010	Rainbow trout	EV-I/CV
23	040915-1/1B	Grønsund (Denmark)	2004	Rainbow trout	NR

*Strain used as bacterin in EV-I

**Strain used as bacterin in EV-II

Further analysis of the target of the serum antibodies in Western blotting showed that EV-I antisera recognized high molecular weight (MW) of purified LPS of two EV-I strains, 090903-1/2B and 100719-1/3A (Fig. 2A, lane 1 and 3). However, low MW LPS bands of the CV strain 850617-1/1 were also detected (Fig. 2A, lane 4). The CV antisera recognized high and low MW LPS bands of two CV strains (090707-1/2A, 850617-1/1), and also the EV-I/CV strain 090819-1/29A (Fig. 2B, lane 2, 3, and 5). Additionally, the low MW section of the LPS of EV-I strain 090903-1/2B (Fig.2B, lane 1) was also detected by the CV antisera. Low molecular weight bands in LPS samples are expected to be associated with the Lipid A and core of the LPS molecule, while intermediate and high molecular weight bands correspond to different sizes of the O-antigen chains [35]. To have representative strains for the Western blotting, the two genetically distant EV-I strains and two genetically distant CV strains were selected based on the phylogenetic tree (Fig. 4).

As a general trend, for EV-I and CV strains, there seemed to be a correlation between strong recognition in ELISA by immune sera of high and intermediate size LPS bands in Western blotting (WB) while antibody binding only low MW LPS bands was not reflected in ELISA reactivity. For the EV-I/CV strain (090819-1/29A), there was no consistent relationship between the serum reactivity pattern against LPS in WB and

against the whole-cell bacteria in ELISA. In this case, only CV antisera recognized LPS of the strain 090819-1/29A, while in the ELISA, it was recognized by both EV-I and CV antisera (Fig. 2A, lane 5, and Fig. 2B, lane 5). Due to the limited availability of the individual immune sera, the immunostaining in Western blotting was performed with sera pooled groupwise. Although individual variations may be expected, the homogeneous reactions in ELISA within the groups suggested that the fish within each group had mounted a relatively similar antibody response.

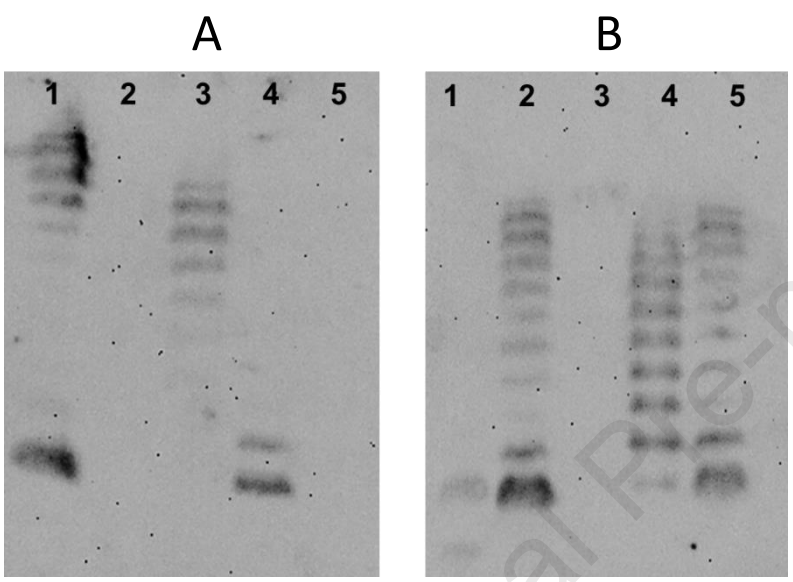


Figure 2. Western blot analysis of VaO2a LPS antigen recognition by trout immune sera. Lane 1: VaO2a 090903-1/2B (EV-I strain), lane 2: VaO2a 090707-1/2A (CV strain), lane 3: VaO2a 100719-1/3A (EV-I strain), lane 4: VaO2a 850617-1/1(CV strain), and lane 5: VaO2a 090819-1/29A (EV-I/CV strain). A: Western blot using a pool of 4 EV-I antisera diluted 1/100 as primary antibody, B: Western blot using a pool of 4 CV antisera diluted 1/100 as primary antibody. None of pooled serum batches recognized the NR strain (040915-1/1B) in Western blotting (data not shown). Sera from the saline group did not recognize any of the LPS samples used in the Western blotting (data not shown).

3.2. Trial I challenge

When the challenge was performed with the strain 090903-1/2B (EV-I strain homologous to the EV-I VaO2a bacterin), only fish given the EV-I were protected (Fig.3A, Table 2 and 3). When the challenge was performed with strain 090707-1/2A (CV strain), both vaccines provided protection (Fig. 3B, Table 2 and 3). However, when the challenge was performed with the strain 090819-1/29A (EV-I/CV strain), neither vaccines were protective (Fig. 3C, Table 2 and 3).

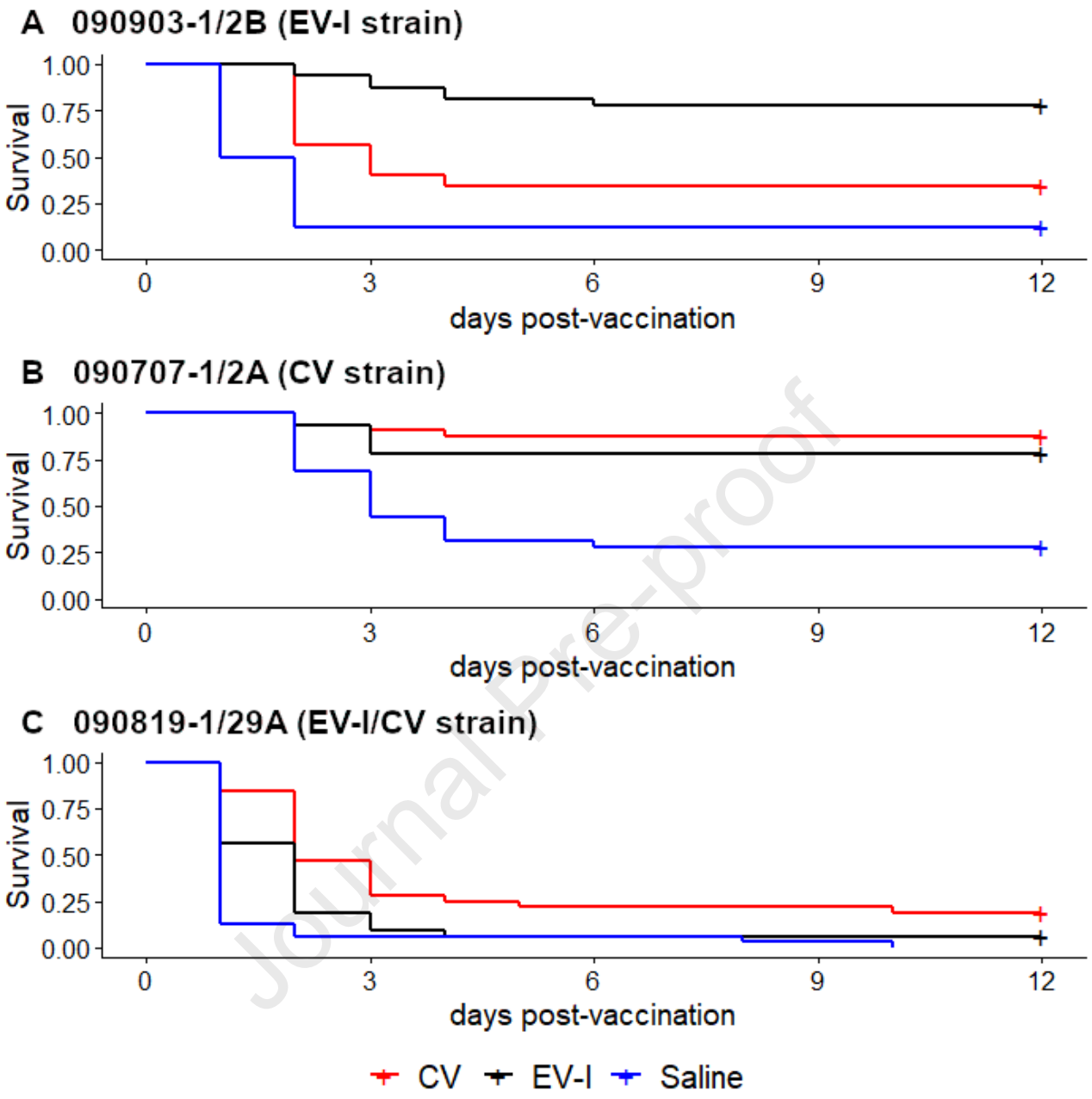


Figure 3. Survival curves of the challenge in trial I. Rainbow trout were IP-injected with either: a commercial vaccine (CV), an experimental vaccine (EV-I), or Saline). At 8 wpv, fish were challenged by IP injection with either *V. anguillarum* strains. (A) Only EV-I group was statistically different from the Saline group (p-value < 0.0001), (B) EV-I and CV groups were statistically different from the Saline group (p-value < 0.001), and (C) None of the groups were statistically different from the Saline group.

Table 2. Survival rates of trial I

	***V. anguillarum* strains used for the challenge in trial I**								
	090903-1/2B EV-I strain			**090707-1/2A CV strain**			**090819-1/29A EV-I/CV strain**		
	Rep. 1	Rep. 2	Average	Rep. 1	Rep. 2	Average	Rep. 1	Rep. 2	Average
CV	37.5%	31.2%	34.4%	87.5%	87.5%	87.5%	18.8%	18.8%	18.8%
EV-I	75.0%	81.2%	78.1%	75.0%	81.3%	78.1%	6.25%	6.25%	6.25%
Saline	6.3%	18.8%	12.5%	31.2%	25.0%	28.1%	0%	0%	0%

The challenge with each VaO2a strain was performed in two replicate tanks (Rep. 1 and Rep. 2) with equal numbers of fish from all groups in each tank. As the differences between the replicate tanks for fish belonging to the same groups were not significant for any group, combined replicates were used for statistical comparison of the groups.

Table 3. Odd ratios of surviving experimental challenge with different Va O2a strains in trial I

Challenge strain	**Treatment**	**Odd ratio of survival**	**Standard error**	**p-value**
090903-1/2B	Saline ↔ EV-I *	25.00	17.11	< 0.0001
	Saline ↔ CV	3.67	2.39	0.1192
	CV ↔ EV-1	6.68	3.87	0.003
	EV-I ↔ CV	0.147	0.083	0.003
090707-1/2A	Saline ↔ EV-I	9.13	5.30	0.0007
	Saline ↔ CV	17.89	11.87	0.0001
	CV ↔ EV-1	0.51	0.349	0.5894
	EV-I ↔ CV	1.96	1.34	0.5894
090819-1/29A	Saline ↔ EV-I	< 0.01	> 1	1
	Saline ↔ CV	< 0.01	> 1	1
	CV ↔ EV-1	0	0	0.322
	EV-I ↔ CV	0	0	0.322

*Example. There was 25 times higher probability of mortality for fish injected with Saline compared to fish injected with EV-I, and the difference was significantly different (p-value <0.0001)

3.3. Correlation between ELISA antigen group and genetic variability of *V. anguillarum* serotype O2a

The full genome analysis showed a minimum of 60 and a maximum of 28.287 SNPs between individual strains, emphasizing the known high diversity within *V. anguillarum* serotype O2a strains [9]. In the phylogenetic tree based on SNPs (Fig. 4), the VaO2a strains tended to cluster according to the recognition in ELISA by the immune sera from vaccinated fish (Fig. 1). The strain 040915-1/1B, which was not recognized by any sera, was most distantly related to the other strains, having from 24.960 to 28.172 SNPs compared to other strains. The EV-I/CV strains clustered together between the EV-I and CV strain groups(Fig.1).

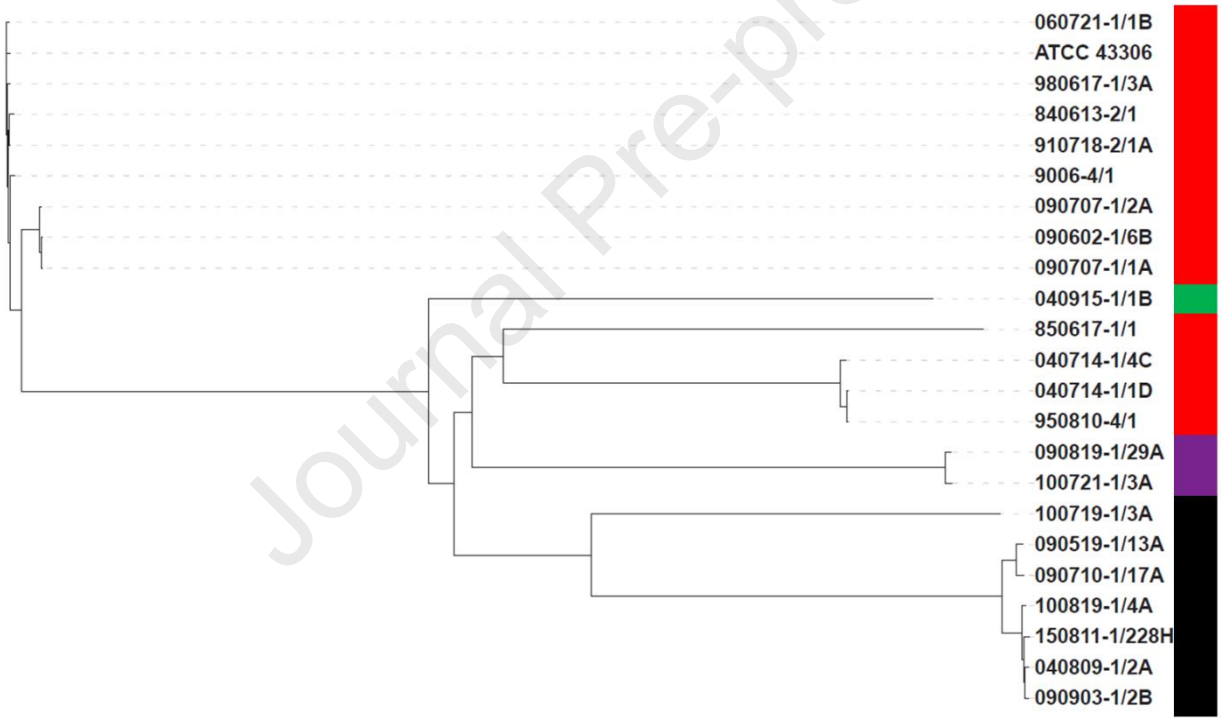


Figure 4. Single nucleotide polymorphism (SNP) tree of 23 strains of *V. anguillarum* serotype O2a. Phylogenetic tree constructed with iTOL v4 based on single nucleotide polymorphisms (SNPs) identified with the pipeline CSI Phylogeny 1.4 using the complete genome of *V. anguillarum* strain VIB12 as a reference. The color bar refers to the serological grouping according to the reactivity in ELISA with sera from vaccinated fish (see Fig.1). EV-I strains (black) and CV strains (red) clustered separately. EV-I/CV strains clustered in between (purple). The NR strain (green) clustered within the CV strains (red group).

When a hierarchical tree based on the presence/absence of genes in the accessory genome was generated, the VaO2a strains also clustered according to the reactivity of vaccinated fish sera, suggesting that the accessory genes could explain the antigen diversity (Fig. 5). The EV-I strains all clustered together, while CV strains clustered into two subgroups. EV-I/CV strains (purple) and the NR strain (green) clustered in between one of the CV strains clusters and the EV-I strains cluster. Considering the 4 sub-grouping (EV-I strains, CV strains, EV-I/CV strains, and NR strain), a comparative analysis was performed to show how many of the accessory genes are unique or shared among the groups (Fig. 6). Statistically, the comparison between CV and EV-I groups is most robust due to the inclusion of more strains in each group. The comparison between the CV strains (n=13) and EV-I strains (n=7) showed that there were 109 unique genes in all CV strains that were absent in all EV-I strains. Conversely, there were 489 genes shared in all EV-I strains that were absent in all CV strains. The single NR strain still shared a considerable number of accessory genes with EV-I strains (308) as well as with the CV strains (123), including some genes (30) shared with EV-1 and CV strains. Information of all genes in the Venn diagram can be found in Supplementary file 1.

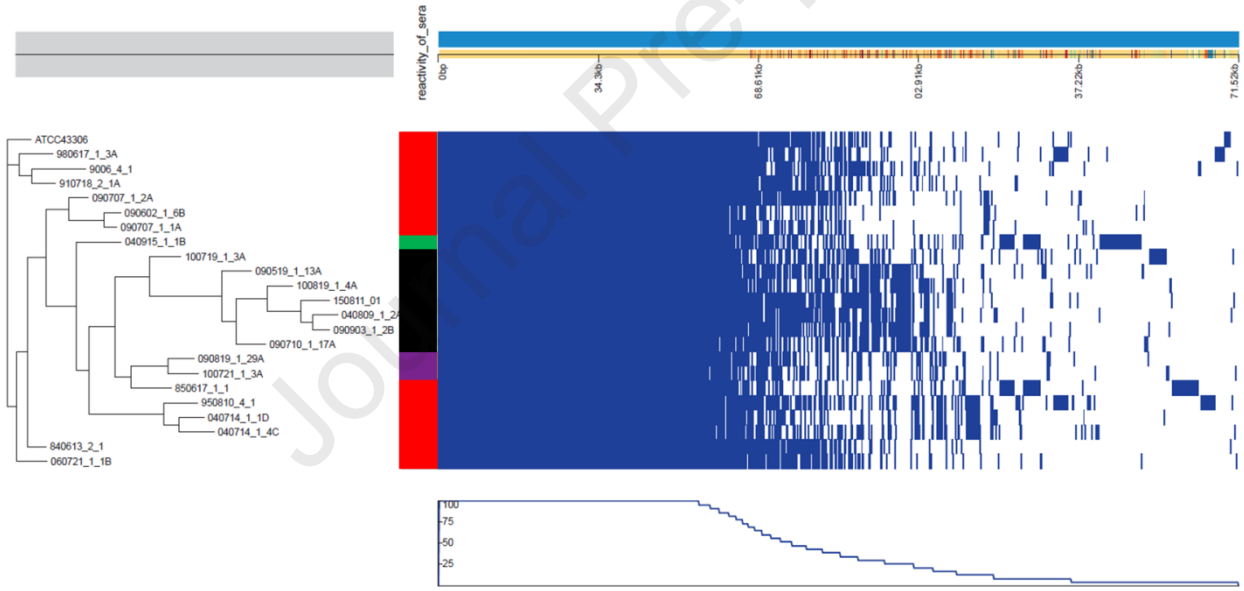


Figure 5. The hierarchical phylogenetic tree of *V. anguillarum* serotype O2a genomes as determined by Roary compared to reactivity of vaccinated fish sera and a matrix with the presence (blue blocks) and absence (white areas) of the accessory genes found in the pan-genome. As in Fig. 4, EV-I strains (black) and CV strains (red) clustered separately. EV-I/CV strains clustered in between (purple). The NR strain (green) did also cluster in between EV-I and CV strains.

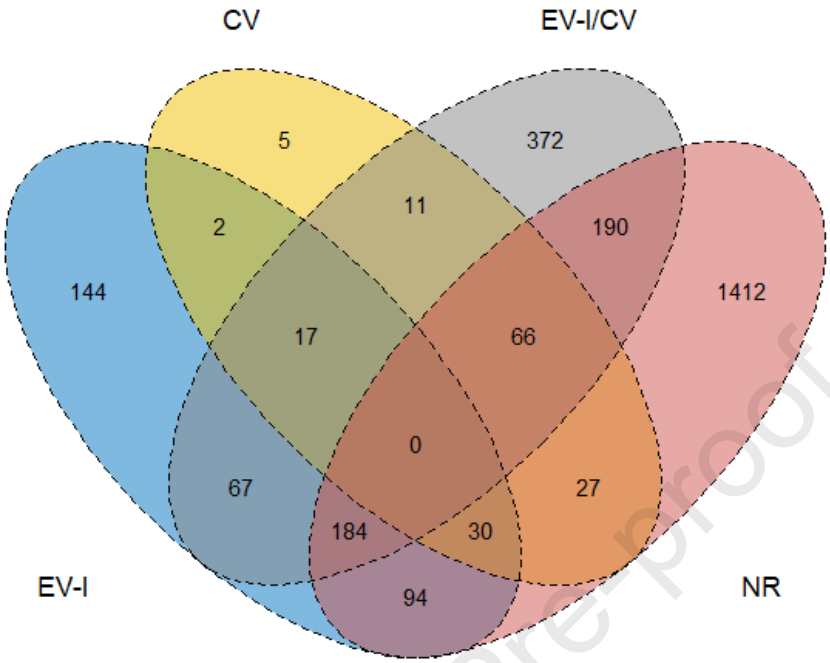


Figure 6. Venn diagram representing the number of shared genes (at intersection) and unique genes (outside intersections) of the accessory genome for each immune serum group: EV-I strains (blue subset), CV strains (yellow subset), EV-I/CV strains (grey subset), and NR strain (red subset)

3.4. Antibody reactivity in sera from fish vaccinated with EV-II

Taking into account that the *V. anguillarum* O2a strain 090819-1/29A, (1) showed a high virulence in the infection trial I, (2) was partly recognized by EV-I as well as by CV antisera in ELISA, and (3) genetically clustered between the EV-I and CV strains, this strain was considered a potential candidate for a cross protective bacterin-based vaccine.

The same setup of the ELISA as in trial I was performed with 10 sera from fish immunized with the EV-II. Immunization with EV-II induced detectable antibodies against all 23 VaO2a strains. Even the most genetically distant strain (040915-1/1B) according to the SNPs phylogenetic tree was also recognized by EV-II antisera, although at a lower level than the other strains. Also, fish given the EV-II mounted a higher antibody response against the vaccine strain (090819-1/29A) than observed for fish vaccinated with EV-I and CV (Fig. 7).

Although all CV and EV-I strains were recognized by sera from the EV-II immunized fish, antibody reactivities and titres were higher against the CV strains (Fig. 7 and 8).

For both trials, antibody reactivities in fish sera were examined by ELISA using only one dilution (1/100) since this revealed a clear distinction between strong and weak recognition of sonicated whole-cell antigens for most VaO2a strains, with surprisingly clearcut reciprocal reaction patterns for the CV and EV-I vaccinated fish. This suggested that the majority of the VaO2a isolates belonged to either of two antigenically distinct subgroups.

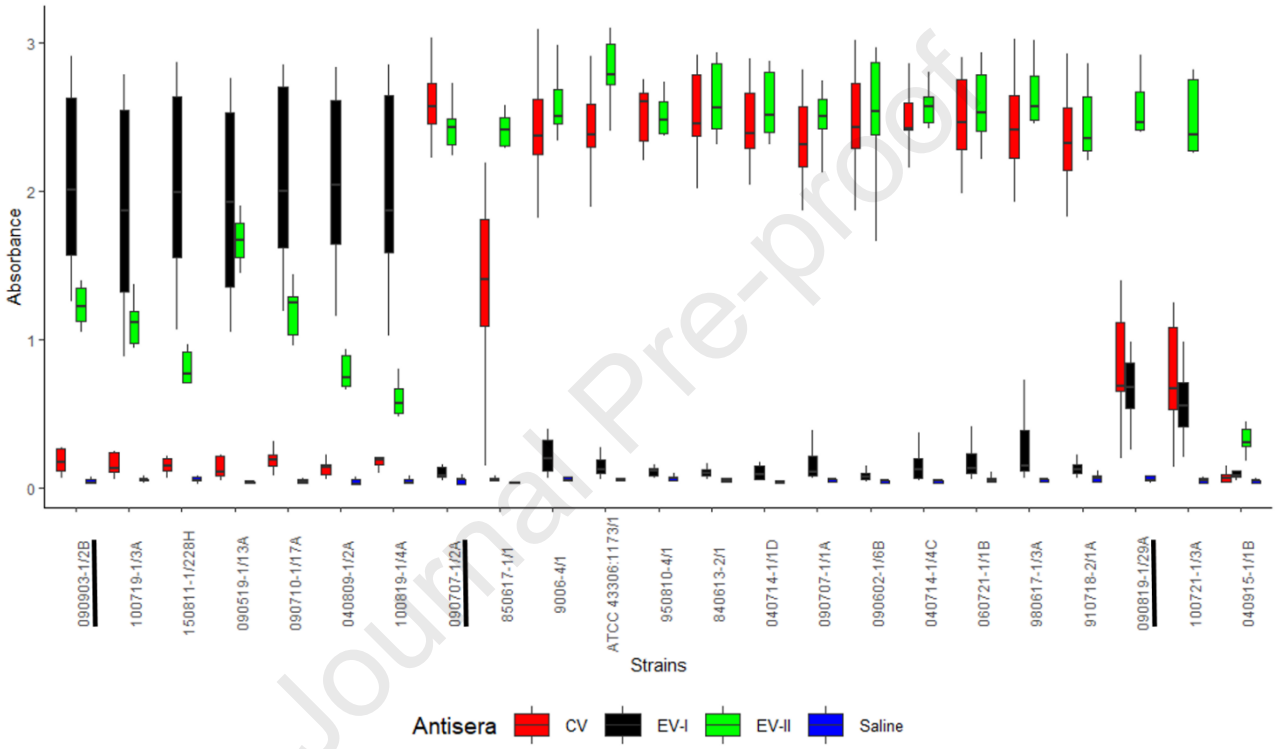


Figure 7. VaO2a strain-specific antibody reactivity of sera from rainbow trout immunized with EV-II (green box, n=10) against 23 strains of *V. anguillarum* serotype O2a (annotated in the x-axis). Results of strain-specific antibody reactivity of EV-I antisera (black box, n=10), CV antisera (red box, n=9), and Saline (blue box, n =3) from the Fig. 1 were used here as a comparative reference. The absorbance scale was normalized in order to combine the data sets of trial I and II. Strains used for challenge in trials I and II are underlined. Sera from fish injected with Saline (trial I) and PBS (trial II) displayed background level reactivity against all bacterial strains.

ELISA results in Fig. 1 and 7 were used to determine whether the individual VaO2a strains were recognized by each antiserum. However, since positive reactions for several strains were in the upper range of the ELISA

readout limit, further differentiation in terms of antibody titers against the different VaO2a strains used in the challenge was approached for the EV-II sera. Vaccinated fish had slightly higher titers of antibodies against the heterologous CV strain (090707-1/2A) than against the vaccine strain (090819-1/29A), while antibody titers against heterologous EV-I strain (090903-1/2B) were 10 times lower (Fig. 8)

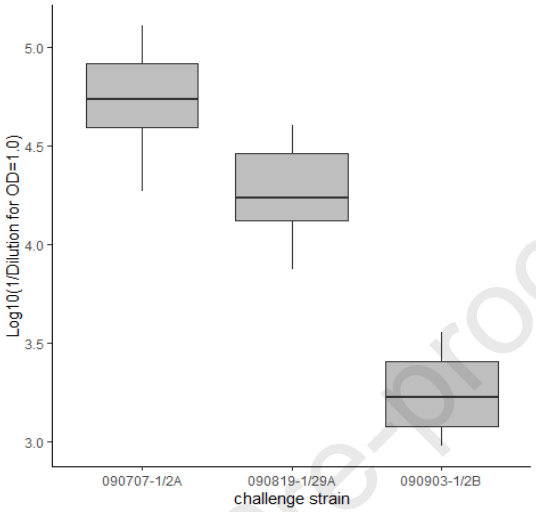


Figure 8. Serum antibody titres of vaccinated fish in trial-II against the VaO2a challenge strains. Serial 10-fold dilutions of sera samples from 10 vaccinated fish were analyzed. Titer was calculated as Log_{10} (1/(Dilution of the sera with O.D = 1.0 in ELISA). All groups were significantly different to each other (p-value <0.001)

In Western blotting, antibodies induced by the immunization with EV-II strongly bound LPS of the homologous strain (090819-1/29A) (Fig. 9, lane 5) as well as LPS of the CV strains (090707-1/2A and 850617-1/1) (Fig. 9, lane 2 and 4). However, no recognition of the LPS of representative of EV-I strains was observed (090903-1/2B and 100719-1/3A) (Fig. 9, lane 1 and 3).

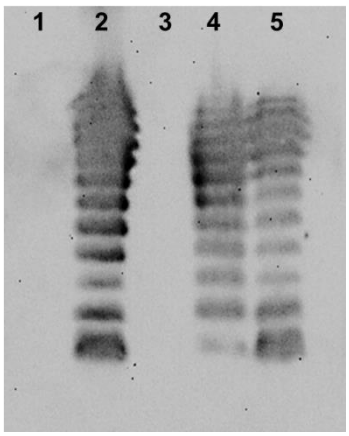
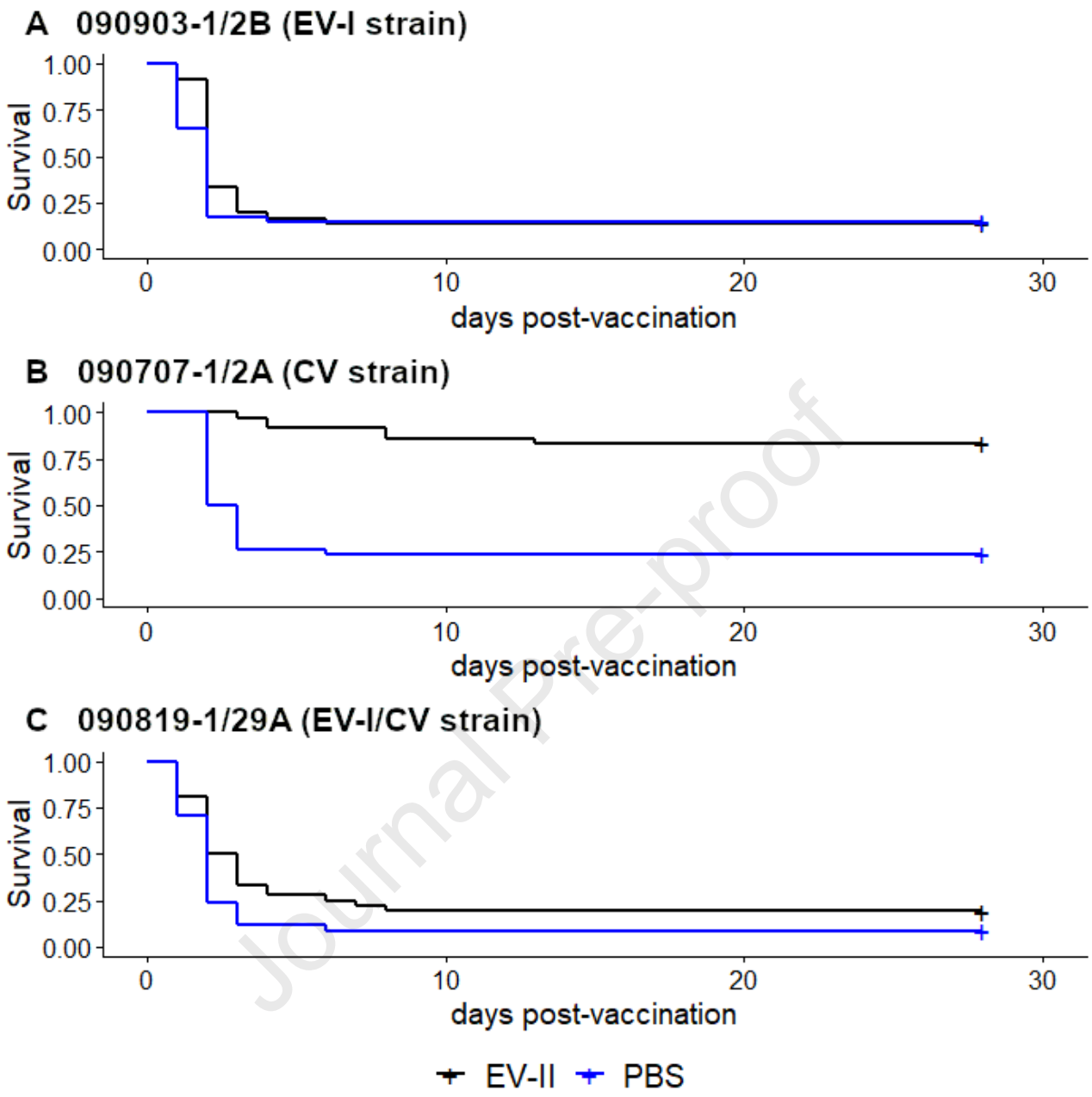


Figure 9. Western blot analysis of VaO2a LPS antigen recognition by trout immune sera. Lane 1: 090903-1/2B (EV-I strain), lane 2: 090707-1/2A (CV strain), lane 3:100719-1/3A (EV-I strain), lane 4: 850617-1/1 (CV strain), and lane 5: 090819-1/29A (EV-I/CV strain). A pool of four EV-II antisera was used as the primary antibody. Sera from the PBS group did not recognize any of the LPS samples used in the Western blotting (data not shown)

3.5. Trial II challenge

The trial II challenge of vaccinated fish was performed with the same *V. anguillarum* strains as used in trial I (090903-1/2B, 090707-1/2A, and 090819-1/29A). The results showed that the EV-II vaccine failed to induce protection against disease caused by the vaccine strain (090819-1/29A) as well as the EV-I strain (090903-1/2B) (Fig. 10). However, the EV-II did protect the fish against the CV strain (090707-1/2A) (Table 4 and 5).



421

422 **Figure 10. Survival curves of the challenge in trial II**. Rainbow trout were IP-injected with an inactivated
423 whole-cell bacterin based on the strain 090819-1/29A (EV-II) or PBS as negative control. The challenge was
424 performed by IP injection with either of three *V. anguillarum* O2a strains. Only when the strain 090707-1/2A
425 was used for the challenge, EV-II group was statistically different from the PBS group (p-value < 0.0001).

426

427

428

Table 4. The survival rate of trial II

	***V. anguillarum* strains used for the challenge in trial II**								
	090903-1/2B EV-I strain			**090707-1/2A CV strain**			**090819-1/29A EV-I/CV strain**		
	Rep. 1	Rep. 2	Average	Rep. 1	Rep. 2	Average	Rep. 1	Rep. 2	Average
EV-II	16.7%	11.1%	13.9%	88.9%	77.8%	83.4%	22.2%	16.7%	19.5%
Saline	5.9%	23.5%	14.7%	23.5%	23.5%	23.5%	11.8%	5.9%	8.85%

The challenge with each VaO2a strain was performed in two replicate tanks (Rep. 1 and Rep. 2) with equal numbers of fish from all groups in each tank. As the differences between the replicate tanks for fish belonging to the same groups were not significant for any groups, combined replicates were used for statistical comparison of the groups.

Table 5. Odd ratios of surviving experimental challenge with different VaO2a strains in trial II

Challenge strain	**Treatment**	**Odd ratio of survival**	**Standard error**	**p-value**
090903-1/2B	Saline ↔ EV-II	0.935	0.639	0.923
090707-1/2A	Saline ↔ EVII	16.3	9.8	< 0.0001
090819-1/29A	Saline ↔ EV-II	2.49	1.84	0.2191

4. Discussions

We here report that the specific reactivity of antibodies induced by bacterin-based vaccines in rainbow trout identified four distinct serological subtypes within a representative panel of *V. anguillarum* serotype VaO2a strains, and that these subtypes correlated with the VaO2a grouping based on the genomic variability. However, vaccine-induced immunity did not consistently comply with the antibody reactivity pattern, and the results further indicated that while strain variations should be taken into account when selecting bacterin-based vaccines, these may still be insufficient for induction of protection against highly virulent VaO2a strains.

The variability of some pathogens, including the ability to change their antigenic epitopes and escape to the host immune response, is one of the major challenges for vaccine efficacy. Vaccines based on conserved and stable antigenic epitopes, like against smallpox, measles, rubella, or diphtheria, induce effective and long-lived acquired immunity against all known strains of the corresponding pathogens. On the contrary, when antigenic epitopes have high variability and low cross-reactivity among strains, vaccines must include several strains in

449 their formulations to have the desired efficacy. In the case of the human disease caused by *Streptococcus*
450 *pneumoniae*, one of the vaccines includes 23 isolates since this pathogen has more than 90 serotypes with little
451 cross-reactivity among serotypes [36–38].

452 Among vaccines for salmonid, those covering *Vibrio anguillarum* (Va) typically include O1 and O2a bacterins,
453 as disease-causing Va strains in Atlantic salmon and rainbow trout usually belong to these serotypes [7,13]. A
454 recent full genome SNP analysis of 44 Va strains derived mainly from vibriosis outbreaks in rainbow trout
455 revealed that among 19 VaO1 strains, 18 grouped in a single homogenous cluster, while 23 VaO2a strains
456 grouped into 7 different clusters. This reflected a much higher genetic heterogeneity among VaO2a strains,
457 mainly due to variability within the accessory genes, i.e. non-shared genes among all VaO2a strains [9]. We
458 recently observed low protection against an assumingly heterologous VaO2a strain and a correlating low
459 antibody reactivity in ELISA in fish vaccinated with a commercial trivalent bacterin-based vaccine (CV), while
460 superior protection in fish given an experimental pentavalent vaccine (EV-I) containing VaO2a bacterin based
461 on the challenge strain correlated with a stronger antibody reactivity with the homologous antigen [10].

462 Taking the newly reported high variability of VaO2a into account [9,39], we here aimed at determining in
463 further detail how genetic and antigenic variability affected the antibody response and protection induced by
464 traditional bacterin based oil-adjuvanted vaccines across a broad panel of VaO2a strains, as well as at
465 identification of a VaO2a variant bacterin able to induce protection across strain variability.

466 When the serum IgM antibody reactivities in vaccinated fish were compared in ELISA across the 23 VaO2a
467 strains using the whole-cells as antigen, the results revealed a clearcut division of the strains into 4 distinct
468 subtypes: Seven strains were strongly recognized only by sera from fish given the EV-I, 13 strains were
469 strongly recognized only by sera from fish given the CV, 2 strains gave an intermediate reaction level with
470 sera from both vaccine groups, and one strain was not recognized by any sera (Fig. 1). The O-antigen used for
471 immunization when preparing mammalian antisera for serotyping is the polysaccharide component of the cell
472 wall of LPS, and it is one of the major immunogenic components of Gram-negative bacteria [7,40]. LPS
473 variability in terms of chemical structure or the length of the O-antigen chain is used as a bacterial mechanism
474 to adapt to the changing surrounding conditions, such as nutrient availability or state of the host immune system
475 [18,41–43]. Our Western blotting (WB) data did support that LPS was one of the key targets recognized by
476 antibodies induced by the bacterin-based vaccines antisera. However, LPS variability could only partly explain
477 the observed antibody reactivity patterns across the examined VaO2a strains. As one example, low molecular
478 weight LPS-bands were sometimes stained in WB even though no antibody binding was observed in ELISA
479 (Fig. 2). Conversely, while EV-I and CV antisera displayed similar reactivities with EV-I/CV strains in ELISA,
480 only CV strains recognized LPS of these strains in WB.

Considering previous reports and our results of antibody recognition patterns (ELISA and WB), and the genetic information (phylogenetic tree) of the VaO2a strains, it is clear that the serotype VaO2a should not be considered as an antigenically homogenous group [13,44]. Additionally, our results support and extend earlier observations suggesting that rabbit antibodies used for serotyping and trout antibodies induced by bacterin-based vaccines recognize different antigenic determinants [45].

The distinction of 4 subtypes of VaO2a by trout immune sera appeared not only to correlate with the phylogenetic clustering of the 23 VaO2a strains in full genome SNP analysis but also with clustering based on the accessory genome variability. Variability in genes involved in the biosynthesis or processing of surface antigens like the LPS could play a role, as these genes have recently been reported to have a high SNP density and gene content variation, which could result in a lack of antibody cross-reactivity [39]. As illustrated by the Venn diagram (Fig. 6) examination of shared and non-shared genes between the 4 subtypes potentially allowed narrowing down the number of genes encoding/involved in the synthesis of antigens recognized by the trout immune sera. The sharp distinction between EV-I and CV strains excluded 49 shared genes, and this was further reduced by 278 genes shared by EV-I strains and the NR strain and 95 genes shared by CV strains and the NR strain. This, in principle, left 144 and 5 unique accessory genes in the EV-I and CV groups, respectively. Similarly, the results identified 17 antigen candidate genes shared by the EV-I, CV and EV-I/CV strains and not found in the NR strain (Fig. 6). In contrast to the antisera induced by EV-I and CV vaccines, the EV-II induced some antibody reactivity with the NR-strain, possibly involving the recognition of antigens related to their 190 uniquely shared genes (Fig. 6). More studies, including detailed characterization/annotation of the variable genes and antibody specificities at protein level are needed to confirm these relations and determine the association between gene variability and antibody response.

In terms of vaccine development, the LPS component of Va has been suggested to represent the key antigen for induction of immunity against vibriosis in salmonids [8,46] and early work by Bøgwald et al.[47] suggested that this was indeed the case for a VaO2 strain in Atlantic salmon. However, Bøgwald et al. reported a lack of correlation between protection and antibody reactivity with whole-cell antigen in ELISA [14], an observation supported by Salati et al. [48]. In contrast, Boesen et al. [49] reported that antibodies (heat-treated immune serum) derived from donors vaccinated with VaO2a bacterin did provide protection against IP challenge by passive immunization. Importantly, while earlier reports on vaccination/challenge trials generally included challenges with the bacterial strain homologous to the vaccine antigen, our results stress the importance of including a panel of heterologous strains for the evaluation of vaccine potency.

Two vaccination trials were included in the animal experiments. In trial I, the relation between antibody reactivity and protection was dependent on the VaO2a strain used for challenge: a direct correlation between serum reactivity (ELISA and WB) and the survival rate was observed when the challenge was performed with the VaO2a EV-I strain (090903-1/2B). The survival rate of fish vaccinated with EV-I was thus 78.1%, while

the survival rate of fish vaccinated with CVwas 34.4%, corresponding to high and low serum antibody reactivity in ELISA, as reported earlier [10] (Fig. 3A). On the contrary, no correlation between immune serum antibody reactivity and survival rate was observed when the challenge was performed with CV strain 090707-1/2A. Fish immunized with CV and EV-I thus had similar survival rates of 87.5% and 78.1%, respectively (Fig. 3B), although the EV-I antisera did not recognize strain 090707-1/2A either in ELISA or in Western blotting.

Since both vaccines failed to protect fish against the highly virulent strain 090819-1/29A, which was intermediately recognized by all immune sera in ELISA (Fig.1), and phylogenetically located between EV-I and CV strains (Fig. 4), we asked ourselves whether bacterin based on this strain (EV-II) would be able to induce protection not only against the homologous strain but also against a broader panel of VaO2a strains. Immunization with EV-II successfully induced antibodies recognizing all strains, but with higher reactivity against the CV and the EV-I/CV strains (Fig. 7). But again, we saw a lack of consistent relationship between the induced antibody reactivities and protection against different VaO2a strains: the EV-II protected against the challenge with the heterologous CV strain (090707-1/2A) but failed to protect fish against the heterologous EV-I strain 090903-1/2B (Fig. 9). While this correlated with high versus intermediate antibody reactivity in ELISA, EV-II induced no protection when the challenge was performed with the homologous vaccine strain, despite high antibody reactivity with this strain in ELISA (Figs 7, 8, and 9).

Low protection against homologous VaO2a IP challenge in bacterin-vaccinated rainbow trout has been reported once before by Santos et al. [50]. Interestingly, these authors found that the same antigen composition delivered by bath vaccination protected turbot against immersion challenge. Mikkelsen et al., (2011) accordingly reported that immunity to immersion challenge with different strains of VaO2 could be obtained in cod following bath vaccination with a mixture of different VaO2 serotypes [12]. The IP challenge route, as applied in our study, bypasses mucosal immune mechanisms and may thus be suboptimal for evaluating whether a vaccine would provide protection under farming conditions. Attempts to challenge 30-50g sized rainbow trout by immersion exposure did not result in sufficient disease to evaluate vaccine efficacy in our hands (data not shown), and we therefore had to rely on challenges by IP injection. Testing of cross-protective effect of bath vaccination of rainbow trout against VaO2a-caused disease could be interesting based on the above mentioned earlier observations, but would have to be done in smaller fish being more susceptible to challenge by immersion. However, the duration of immunity following bath vaccination is usually shortlived, and as we here focused on vaccines aiming at induction of protection for 1½-2 years, as needed in the 3-year production cycle for sea-reared rainbow trout in Denmark, early bath/dip vaccination would most likely not be sufficient alone.

Taking the high genetic variability among the examined VaO2a strains into account, it cannot be excluded that protective mechanisms also vary between isolates dependent on antigen composition and virulence. Further

549 analyses are thus needed to determine whether antibodies against LPS might provide protection against some
550 strains, e.g. low-virulence strains with LPS structures similar to the vaccine strain. Similarly, non-LPS related
551 protection could reflect that cell-mediated immunity was more important than humoral immunity or that the
552 antibodies targeting non-LPS antigens play an essential role in the protective mechanism.

553 The reason for the lack of protection against homologous challenge with the VaO2a 090819-1/29A strain
554 despite a strong antibody response could be several. One possibility is that some VaO2a strains express other
555 antigen variants /virulence components *in vivo* compared to when cultured *in vitro* in conventional media. It
556 has been observed that LPS-antigens changed to some extent when fish serum was added to the medium, and
557 it might be expected that larger differences would occur in comparison of *in vitro* and *in vivo* growth [18]. The
558 fact that the VaO2a 090819-1/29A strain together with one other strain (100721-1/3A) grouped separately
559 from the two larger VaO2a clusters in the hierarchical analysis of the accessory genome, supports the idea that
560 these isolates either harbour genes not shared with most other VaO2a strains, or possibly lack genes encoding
561 vaccine antigens/epitopes (Figs. 5 and 6). An earlier comparative analysis of the occurrence of various
562 virulence markers among the 23 VaO2a strains included in the current study did in fact, indicate that the two
563 isolates carried a unique virulence marker composition when taking all 52 included markers into account [9].
564 The two variant strains also carried the p15 plasmid, which might have contributed to their unique accessory
565 genes and antigen composition, although not encoding any predicted virulence markers [9]. Further
566 examinations, including comparative proteomics of VaO2a strains grown under different culturing conditions,
567 will be needed to address these aspects.

568 In recent years, most outbreaks of vibriosis in farmed salmonids have been associated with VaO1 variants
569 (unpublished observations), and it may be argued that the inclusion of VaO2a antigen/bacterin in the vaccine
570 is less critical [51]. However, the exchange of genetic elements between Va isolates was recently described,
571 and it appears to be a relatively common event, potentially including serotype switching [39]. Interestingly,
572 Hansen et al. identified an aberrant VaO1 isolate grouping with VaO2a in the pan-genome SNP phylogenetic
573 analysis. That strain (VaO1 090819-1/28A) was isolated from the same disease outbreak in sea-reared rainbow
574 trout as the high virulent VaO2a 090819-1/29A strain included in the current study and carried a partly similar
575 virulence marker composition – excluding the p15 plasmid. Although this may be a coincidence, the co-
576 occurrence of VaO1 and VaO2a stresses the importance of taking local variants of both VaO1 and -O2a into
577 account when selecting the vaccine. Possibly, autogenous vaccine tailoring according to VaO2a variants could
578 be superior to using commercially available vaccines.

579 Still, our results with poor protection against homologous challenge for the VaO2a 090819-1/29A strain
580 suggest that other approaches than traditional bacterin-based vaccines might be required for efficient protection
581 of rainbow trout against some VaO2a variants. These might include recombinant protein- or DNA-based

vaccines, which both have been reported to provide significant protection against vibriosis, although in other fish species [52].

In summary, our results demonstrated that the VaO2a bacterin induced antibody response in rainbow trout dependent on the strain used for immunization and that the VaO2a strains could be grouped into at least 4 different subtypes based on reactivity with trout immune sera. Although antibodies against the LPS antigen might provide protection against some, possibly low virulence and genetically similar VaO2a strains, there was no consistent relationship between protection and antibody response to neither whole-cell antigen nor the LPS fraction. The lack of cross-protection between different isolates suggested that a vaccine must be selected/formulated according to the locally prevalent VaO2a strain variants. Genetic, antigen – and virulence typing (assays) based on SNP markers should be developed for strain characterization. And in the case of occurrence of certain high virulent variants, traditional bacterin-based vaccines may not provide efficient protection. A better understanding of protective immune mechanisms along with the development of alternative recombinant protein- or DNA–based vaccines might be required to prevent vibriosis caused by such strains.

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Declaration of Interest

The authors declare no conflict of interest

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Highlights

- High antigenic variability was observed among strains of *Vibrio anguillarum* O2a
- Limited cross-reactive antibody response was induced by two Vibrio bacterins
- Antibody response does not consistently correlate with protective response
- Bacterins can induce protection even though a low antibody response was observed