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Effect of oral booster vaccination of rainbow trout against *Yersinia ruckeri* depends on type of primary immunization

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Abstract

Vaccination of rainbow trout against Enteric Redmouth Disease (ERM) caused by *Yersinia ruckeri* can be successfully performed by administering vaccine (a bacterin consisting of formalin killed bacteria) by immersion, bath or injection. Booster immunization is known to increase the protection of fish already primed by one of these vaccination methods. Oral vaccination of trout (administering vaccine in feed) is an even more convenient way of presenting antigen to the fish but the effect of an oral booster has not previously been described in detail. The present work describes to what extent protection may be enhanced by oral boostering following priming with different administration methods. The study confirms that vaccination by 30 sec dip into a bacterin (diluted 1:10) may confer a significant protection compared to non-vaccinated fish. The immunity may be optimized by booster immunization either provided as dip (most effective), bath (less effective) or orally (least effective). Oral immunization may be used as booster after dip but applied as a single oral application it induced merely a slight and statistically non-significant response. It is noteworthy that primary oral immunization followed by an oral booster vaccination showed a trend for an even weaker response. It should be investigated if continued exposure to a low antigen concentration – as performed by two oral immunizations - may induce tolerance to the pathogen and thereby leave the fish more vulnerable.

Key words: Vaccination, oral, dip, bath, *Yersinia ruckeri*, IgM

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Introduction

Enteric Redmouth Disease ERM is caused by the enterobacterium *Yersinia ruckeri* and is considered one of the most problematic diseases leading to severe economical losses in freshwater farming of salmon and trout [1]. Clinical signs are bleedings in the mouth, surface tissues (particularly fins and fin bases) and at necropsy hemorrhages are also detected in internal organs including the gastro-intestinal tract [2, 3]. The disease can be treated by use of various antibiotics but already decades ago vaccination methodologies were tested with some success in traditional trout farms [4-6]. These classical immunoprophylactic studies were performed with the use of formalin killed *Y. ruckeri* bacteria (bacterin) and administration method studies showed that the way of presenting *Y. ruckeri* antigens to rainbow trout has an important effect on the protective response. Intraperitoneal vaccine injection led to a superior effect compared to dip of trout into the bacterin [5, 7]. However, protection conferred by dip vaccination may be further improved by booster vaccination either by re-dipping for 30 sec or by bathing fish in a diluted bacterin for at least one hour [8, 9]. Still injection and dip vaccination procedures are time consuming and if the antigens could be supplied with the fish feed it would reduce handling of fish and labor during vaccination procedures. Oral presentation of *Y. ruckeri* antigens to trout has been tested previously [10, 11] but the protection conferred was generally low. However, oral booster vaccination has been suggested as a way to increase protection, and a commercial vaccine (AquaVac ERM oral, MSD Animal Health) is available, but at present no precise evaluation of this administration method compared to dip or bath is available. We have therefore performed a comparative study in order to determine the relative protective effect of booster vaccination using dip, bath and oral antigen administration. Antibody reactions in the fish were followed by measuring IgM in sera from fish after vaccination and after challenge with live *Y. ruckeri* bacteria.

Materials and Methods

Fish

A total of 3000 rainbow trout were produced by hatching disinfected trout eggs (7-8 °C) of the Rakkeby strain (Jutland, Denmark) and subsequent rearing (12-13 °C) at the Salmon Hatchery, Bornholm, Denmark, which is a pathogen-free recirculated facility supplied with municipal freshwater [12]. Fish were kept in 14 tanks (1 m^3 tanks each containing 700 L municipal freshwater) and fed (1% biomass) with commercial standard pelleted feed (Biomar A/S, Brande, Denmark).

Experimental groups and vaccination

A more comprehensive outline of the schematic overview of the experimental setup can be found in the supplementary materials (Fig. S1). Primary vaccination was performed using 6 months old fish (2160 degree days) with a mean body weight of 3.5 g. The experimental set-up comprised group 1: A total of 1000 rainbow trout kept as unvaccinated control fish, group 2: A total of 1000 rainbow trout immunized by oral vaccination (AquaVac ERM oral, MSD Animal Health) by supplying vaccine (0.1 ml/fish) in feed (1% biomass per day) over 2x5 days with a break of 5 days in between according to the manufacturer's recommendations and group 3: A total of 1000 rainbow trout dip vaccinated for 30 s with AquaVac® RELERA (MSD Animal Health) vaccine (diluted 1:10) containing formalin-inactivated *Yersinia ruckeri* biotype 1 (5×10^9 cells/ml) and *Y. ruckeri* (EX5) biotype 2 (5×10^9 cells/ml). All fish groups were then kept separately in 1 m^3 fish tanks (water volume 700 l). Booster vaccination was conducted 45 days post-primary vaccination and four additional groups were hereby formed and comprisedgroup4: A total of 200 fish orally vaccinated once was orally boostered by giving 0.1 ml vaccine (AquaVac® ERM oral) /fish in feed (2x5 days with a break of 5 days on normal feed in between), group 5: A total of 200 fish dip vaccinated once were boostered by dip into bacterin (AquaVac® RELERA) diluted 1:10 for 30 sec, group 6: A total

of 200 fish vaccinated by dip once was orally booster vaccinated by using AquaVac® ERM oral as described above, and group 7: A total of 200 fish dip vaccinated once were boostered by 1 h bath (bath vaccination defined as long term exposure to diluted antigen for 1 hour or more) in diluted bacterin (1:1000) (AquaVac® RELERA). Control fish were sham vaccinated by immersion into pure water and by feeding them with commercial standard pelleted feed (Biomar A/S, Brande, Denmark). Thus a total of seven duplicated groups (14 fish tanks) were treated (Table 1).

Fish infection facility

For the challenge experiment subsamples of fish from all groups were transported to the fish infection facility at the University of Copenhagen, Frederiksberg, Denmark, where they were exposed to live bacteria. At arrival the pathogen free status of fish was confirmed by examining two fish from each group by standard bacteriological and parasitological examination [13]. Fish were acclimatized at 14°C for 14 days before experimentation and fed (1% biomass) with commercial standard pelleted feed (BioMar A/S, Brande, Denmark).

Challenge experiments

The challenge experiment was performed on day 97 (1358 degree days) post-primary vaccination by exposing (using i.p. injection) 3 subgroups of each treatment to three different dosages of *Y. ruckeri* serotype O1 biotype 2 (100415-1/4): 1) low dosage (6.5×10^4 cfu/fish), 2) medium dosage (6.5×10^5 cfu/fish) and 3) high dosage (6.5×10^6 cfu/fish), respectively. The *Y. ruckeri* strain used was isolated from a Danish fish farm [14] and further characterized according to Wheeler et al. [15]. All exposures were performed in duplicate (2x25 fish). Experimental fish were anesthetized with MS222 (50 mg/l) and live bacteria were administered by intraperitoneal injection of 100 µl bacterial suspension. After administration of the live bacteria groups of challenged fish were returned to individual fish tanks (water volume 100 l). Fish were observed with 2 h intervals following challenge and moribund fish were removed, and euthanized in an overdose (300 mg/l) of MS-222

(Tricaine methane sulphonate, Sigma-Aldrich). *Y. ruckeri* O1 biotype 2 was re-isolated from head kidney of freshly euthanized fish (2 fish/group) on blood agar plates to confirm that the disease was caused by the challenge strain [14].

Sampling

Blood sampling was conducted by caudal vein puncture (ten samples per group) at three time points: at 52 days (728 degree days) and 90 days (1260 degree days) post primary vaccination/ 45 days post booster (45 dpb) (before exposure to live bacteria) and at 3 weeks post-challenge (from fish exposed to a low dosage of *Y. ruckeri*) corresponding to 119 days/ 74 dpb (1666 degree days) after the primary vaccination. Blood was kept at 4 °C overnight, centrifuged at 3000 g for 10 min (4 °C), and serum separated and stored at -80 °C until measurement of specific antibody levels against *Y. ruckeri*.

Antibody analysis

Specific antibody in fish raised against *Y. ruckeri* was measured by ELISA according to Chettri et al. [7, 16]. In brief, a 48 h *Y. ruckeri* O1 biotype 2 (100415-1/4) culture (LB culture medium) was used for coating microtiter plates. Plates were washed three times in washing buffer (PBS pH 7.4 with 0.1% Tween 20) and non-specific binding sites were blocked by incubation with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Serum samples from all groups were analyzed in duplicate wells. A diluted serum sample (100 µl, diluted 1:50 and 1:100) was added to the plate and incubated 12 h at 4 °C. After 3 times wash each well was incubated with mouse anti-salmonid Ig (dilution 1:500; AbD Serotec Cat. MCA2182, Dusseldorf, Germany) for 1 h at room temperature on an orbital shaker (50 rpm) which was followed by 3 times washing and incubation with HRP (horseradish peroxidase) conjugated rabbit anti-mouse IgG (dilution 1:500; AbD Serotec Cat. STAR13B, Dusseldorf, Germany) for 1 h at room temperature as above. Chromogenic substrate TMB (AbD Serotec Cat. No. BUF042B, Dusseldorf, Germany) was then added, the

reaction stopped after 15 min by adding 1N HCl and OD read at 450 nm in an Epoch spectrophotometer (Biotek Instruments, Inc. Winooski, USA).

Statistical analysis

Normality test (Shapiro-Wilk) was conducted for the evaluation of the normal distribution of data within each group. The software GraphPad Prism 5 was used for creating graphs and performing statistical analysis. Mortality data from the infection experiment were analyzed by the Kaplan-Meier test and differences among groups were tested using one way ANOVA with Tukey's post hoc test after combining data from the replicates. ELISA data are presented from 1:50 serum dilutions and differences in antibody level (OD) between groups were tested using Student's *t*-test and Mann–Whitney *U* test. Groups were considered significantly different when $P < 0.05$.

Results

Survival following challenge

No significant differences were found between duplicate tanks, which allowed pooling of mortality counts from these for further analysis. In order to detect the vaccine effect, the challenge dosage was adjusted at three different levels which elicited different mortality rates in vaccinated fish. The best survival rate was seen in fish vaccinated twice by dip vaccination (2 x dip). Fish receiving the low challenge dosage showed a higher survival rate compared to fish challenged with medium and high dosages. Survival rates were 97% and 92% in group 5 (2 x dip) and 7 (1 x dip + 1 x bath), respectively. Fish immunized once by 1 x dip and twice by 1 x dip + 1 x oral showed survival rates of 69% and 68%, respectively. Non-immunized control fish showed a survival rate of 49% which was not significantly different from fish exposed to 1 x oral and 2 x oral (44%) (Table 2) (Fig. 1A). Fish challenged with the medium dose exhibited 83%, 56%, and 49% survival (groups exposed to 2 x dip, 1 x dip + 1 x bath, and 1 x dip fish), respectively. The other vaccinated fish showed 31%, 28%, 11%, and 5 % survival rates (1 x dip + 1 x oral, 1 x oral, control, and 2 x oral), respectively

(Table 2) and (Fig. 1B). Fish injected with the high dose of *Y. ruckeri* exhibited a relatively high mortality even in vaccinated fish. Non-immunized control fish and fish receiving 2 x oral vaccination exhibited comparable survival rate 5 % and 6 %, respectively. Fish dip immunized once (1 x dip) and orally immunized once (1 x oral) showed survival rates of 11 and 13%, respectively. Fish vaccinated once by dip and boostered orally (1 x dip + 1 x oral) or boostered by bath (1 x dip + 1 x bath) exhibited survival rates of 22 % and 28 %, respectively. Fish dip vaccinated twice (2 x dip) showed the best resistance with a 40 % survival rate (Table 2) and (Fig. 1C).

Antibody responses measured by ELISA

At 52 days post-vaccination (pre-challenge) no significant differences were found with regard to antibody (IgM) levels between control, one time dip vaccination (1 x dip), and one time oral vaccination (1 x oral) (Fig. 2A). Samples from fish taken 45 days after boostering (but before challenge) showed a slight but significant elevation of antibody levels in all groups which had received booster vaccination (2 x oral, 2 x dip, and 1 x dip + 1 x bath), except of fish which had received 1 x dip + 1 x oral, while control and one time vaccinated fish (1 x oral and 1 x dip) showed no change of antibody levels (Fig. 2B). Three weeks post-challenge (74 dpb) the antibody levels were elevated in all groups. In general, all groups exhibited higher antibody level compared to the level before challenge. Only two groups (1 x dip + 1 x oral) and (1 x dip + 1 x bath) exhibited significantly higher antibody responses compared to challenged but previously naïve (non-vaccinated) fish (Fig. 2C).

Discussion

It is generally recognized that the administration methods applied when vaccinating fish play a crucial role for the protection induced [5]. Injection vaccination provides a superior protection of rainbow trout towards *Yersinia ruckeri* compared to dip (short term immersion into a high concentration bacterin) and bath (long term bath in a low concentration bacterin) [16]. If adjuvants are combined with the vaccine antigens the protection after injection vaccination can be even higher [7]. However, small fish with a body weight of a few grams are not easily handled for injection vaccination and alternative administration methods or booster immunization may instead be optimized in order to achieve higher protection [5]. Thus, boostering by dip or bath has been shown to be effective both in the laboratory and in the field [8, 9]. Oral administration of vaccine may be an additional way to administer booster immunization but the precise effect of this methodology has remained unclear. The present investigation elucidated to which extent ERM vaccine antigens provided in fish feed confer immunity of rainbow trout against *Y. ruckeri* infection. A primary oral vaccination of small rainbow trout - by feeding each fish over 2 × 5 days with a total of 0.1 ml vaccine in feed - only interrupted by a 5 day break - gave the fish a slight (statistically insignificant) protection against *Y. ruckeri* infection. However, if used as booster vaccination – after a primary dip vaccination in a bacterin diluted 1:10 for 30 sec - the oral administration provided led to a slightly increased protection when exposed to a medium or a high concentration of live *Y. ruckeri* bacteria. When an effective primary immunization against ERM has taken place it seems that boostering by the oral route may lift the response slightly. However, the study did also show that oral vaccination may not always be beneficial. If oral administration was used twice – without any prior strong immunization – a trend for a slightly (although statistically non-significant) decreased disease resistance (elevated mortality after challenge) was noted. It cannot be excluded that the low protection following two times oral antigen administration may be caused by induction of tolerance

to the pathogen. Induction of tolerance is a basic mechanism in the immune system [17, 18] as responses in a host against some antigens including self-antigens are clearly devastating. Therefore such auto-reactions must be avoided and during the ontogenetic development of the immune system the host organism exerts clonal deletion of self-reactive lymphocyte clones [19]. In addition, host reactions against microbiotas (associated with the skin, gills and gut) must be tightly regulated as exacerbated inflammatory reactions would be highly problematic as there is a constant bacterial exposure of outer or inner mucosal surfaces. Thus, the highly debilitating condition IBD (Inflammatory Bowel Disease), which is widespread in the industrialized part of the world, is caused by a severe inflammatory reaction of the host intestinal immune system against even benign gut microorganisms [20, 21]. Therefore, the host must be able to activate and apply immunological pathways in order to achieve tolerance to certain microorganisms which are recognized as non-harmful, unable to penetrate the host or occur in that low densities that they are not perceived a danger by the host. These regulatory immune mechanisms may explain the inferior efficacies achieved after dermal vaccination against tuberculosis in certain areas where the human population is continuously exposed to environmental mycobacteria [22]. Correspondingly, mice orally immunized against antigens may exhibit a depressed reaction and immunity development towards these specific antigens [23]. Tolerance may include the action of regulatory T-cells with depression of cytokine expression [24] and possibly other immune mechanisms. It can therefore not be excluded that oral vaccination against ERM using feeding with a low concentration of formalin killed *Y. ruckeri* will initiate a state of immunological memory in rainbow trout but due to the evidently non-pathogenic nature of the killed antigens, combined with the extremely low concentrations, the tolerance pathway may be activated. To what extent the rainbow trout immune system applies Treg cells, immune regulating cytokines or other mechanisms in this process should be further investigated. It is generally found that immersion of fish into a bacterin merely elicits a

very low antibody reaction [25] and we have previously illustrated that antibody levels in sera were elevated post-vaccination particularly in injection and twice immersion vaccinated fish. In the present study we saw merely a light (but statistically significant) elevation of Ig levels in boostered trout which were best protected groups but this may point to the importance of antibody based protection as corresponding to previous work on the role of humoral immunity of rainbow trout in elimination *Y. ruckeri* [7, 8]. In addition, we showed in a previous study that the densities of IgM-positive lymphocytes in trout spleens were significantly higher in fish immersed three times in bacterin compared to control fish [26]. In the present study the ELISA data showed that some immunological priming must have taken place as exposure to the live *Y. ruckeri* induced different antibody elevations in the differently primed groups – including fish immunized orally twice. Therefore protection is not solely reflected by antibody levels but must also involve various cellular reactions and regulatory mechanisms. This notion is supported by previous studies indicating that cellular mechanisms contribute to immunity against *Y. ruckeri*. Thus, expression levels of rainbow trout immune genes including T-cell markers (TcR, CD8α and CD4) were elevated in spleen tissue of rainbow trout dip-vaccinated against *Y. ruckeri* [27]. Further, expression levels of genes encoding $CD4^+$ T (T-helper/TH), T_H1, T_H2, T_H17, Tregs, and $CD8^+$ T (cytotoxic T lymphocytes/CTLs) markers were up-regulated in challenged fish groups [7]. This indicates that protection of trout is regulated by an intricate network of immune factors in accordance with previous studies on vaccinated salmonids [28, 29, 30].

In conclusion the present study confirmed that dip vaccination combined with boostering by dip, bath or oral antigen presentation will elevate the protection against *Y. ruckeri*. However, it is noteworthy that a primary oral immunization followed by a corresponding oral booster left trout as susceptible as naïve control fish.

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Tables:

Table 1. **Treatment groups**. Seven duplicated groups were formed. Fish were immunized by oral and dip vaccination. Oral vaccination (AquaVac ERM oral) was conducted by administrating vaccine 0.1 ml/fish in feed (1% biomass per day) 2x5 days with a break of 5 days in between according to the manufacturer's recommendations. Dip vaccination was performed by vaccinating fish for 30 s with AquaVac® RELERA (MSD Animal Health) vaccine (diluted 1:10) containing formalin-inactivated *Yersinia ruckeri* biotype 1 (5×10^9 cells/ml) and EX5 biogroup *Y. ruckeri* biotype 2 (5×10^9 cells/ml). Control fish were sham vaccinated by dipping into pure water or feeding them with commercial standard pelleted feed (Biomar A/S, Brande, Denmark).

Tabel 2. Statistics of survival data presented in fig. 1. In order to elucidate differences between the treatment groups one way ANOVA with Tukey's post hoc test was conducted. NS: no significant difference. *: $p<0.5$, **: $p<0.01$, ***: $p<0.001$.

Figures:

Figure 1: Percentage survival (Kaplan-Meier) of rainbow trout in different experimental groups following challenge 97 days post-1st vaccination by three different doses (low, medium, and high) with *Yersinia ruckeri* O1 biotype 2. Differences among groups were tested using one way ANOVA with Tukey's post hoc test after combining data from the replicates. A: Low dose (6.5×10^4 cfu/fish). B: Medium dose (6.5×10^5 cfu/fish). C: High dose (6.5×10^6 cfu/fish).

Figure. 2. *Yersinia ruckeri* specific antibody (IgM) levels measured by ELISA (enzyme-linked immunosorbent assay). Each symbol represents a single fish (total of 10 fish/group were analyzed) and average antibody level in each group is shown by horizontal line. A: Pre-challenge (52 days post-1st vaccination). B: Pre-challenge (97 days post-1st vaccination/ 45 days post-booster). C: Three weeks post- exposure to live bacteria (126 days post-1st vaccination/ 74 days post-booster). Student's *t*-test and Mann–Whitney *U* test (*, $P < 0.05$).

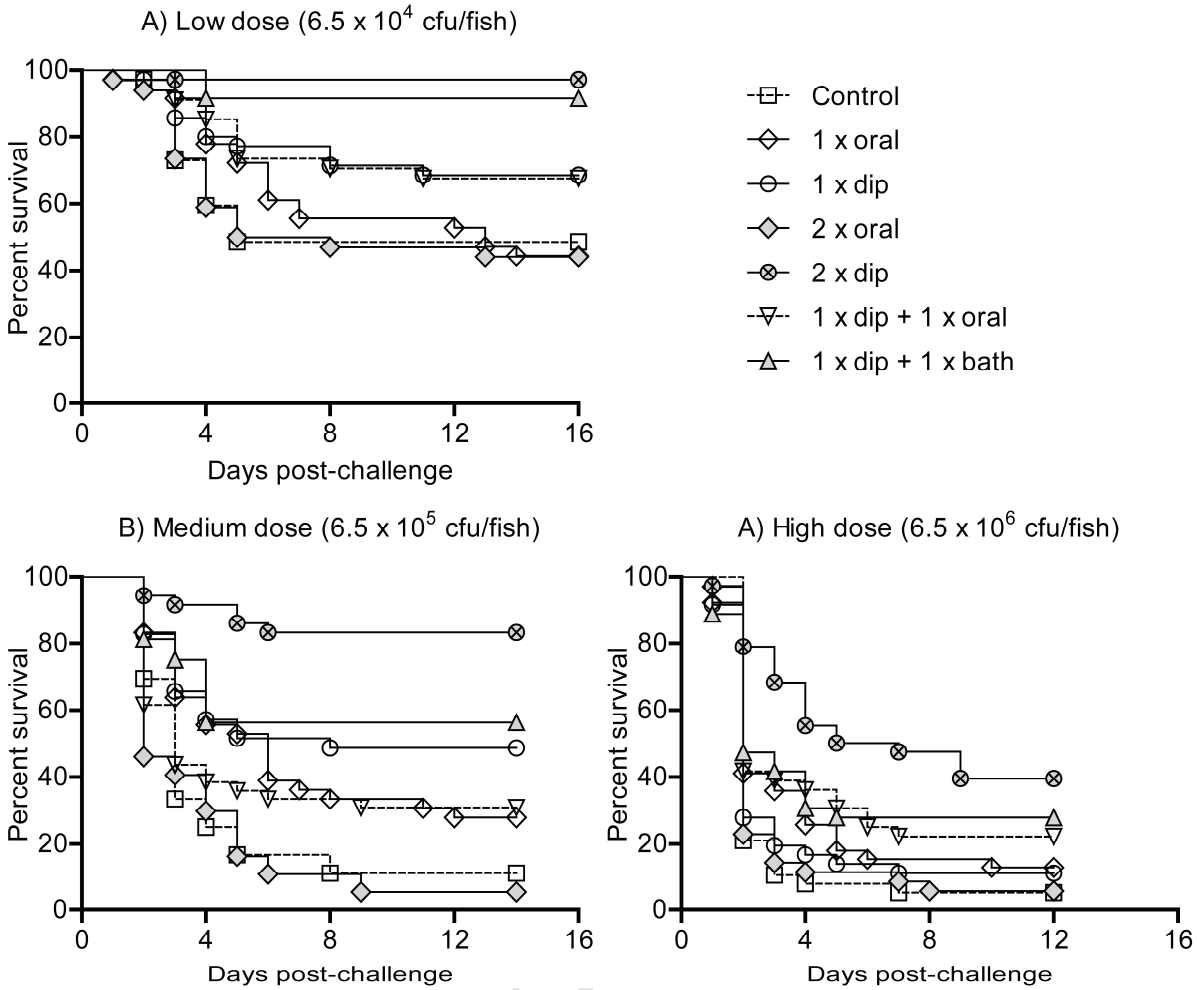
Supplementary figure: Schematic overview of the experimental setup.

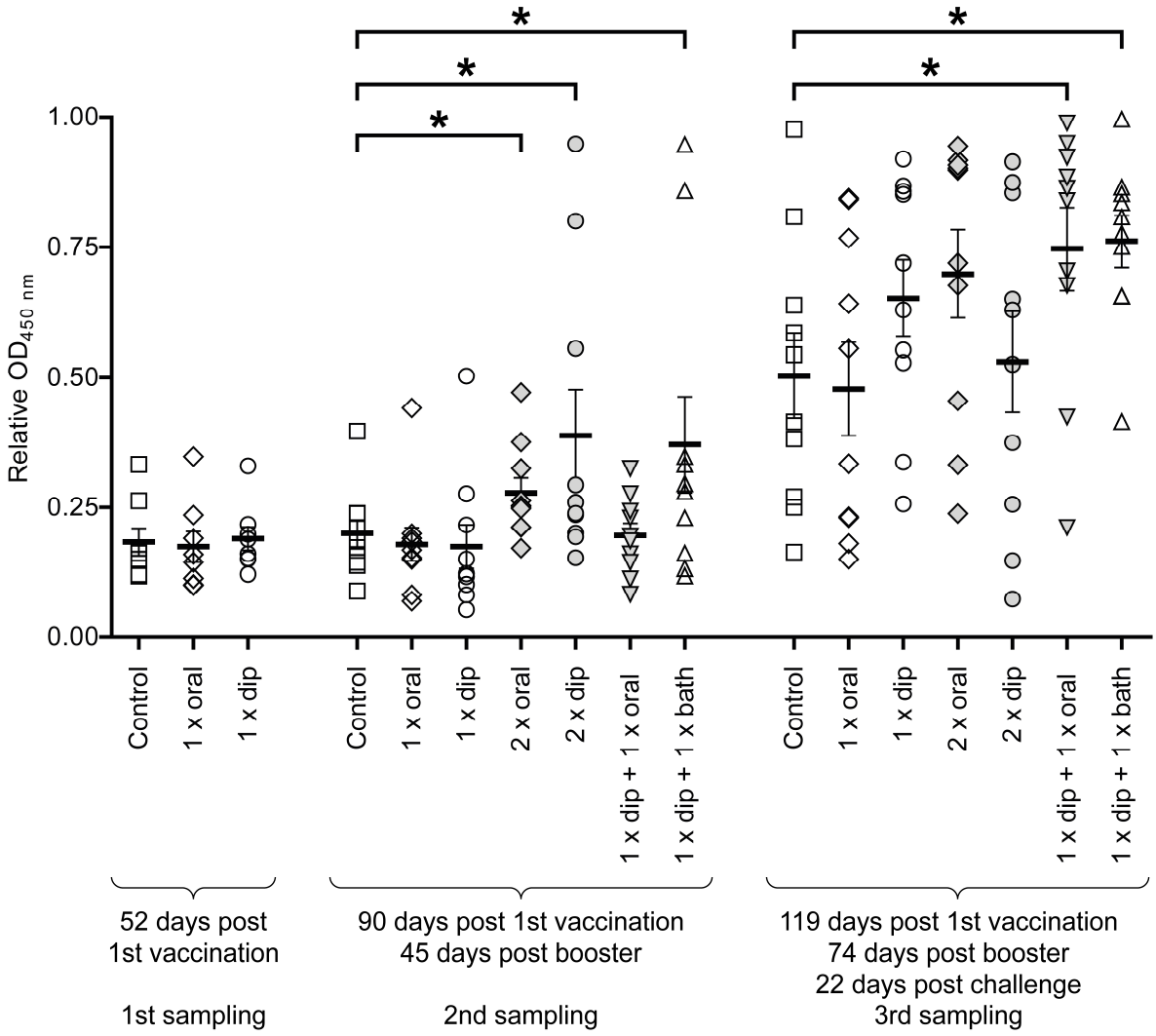
Table 1

Group no.	Treatment	Group ID
1	Sham vaccinated control (No vaccination at all)	Control
2	One time oral vaccination (AquaVac ERM oral)	1 x oral
3	One time dip vaccination (AquaVac® RELERA)	1 x dip
4	Two times oral vaccination (AquaVac ERM oral)	2 x oral
5	Two times dip vaccination (AquaVac® RELERA)	2 x dip
6	One time dip vaccination + One time oral vaccination	1 x dip + 1 x oral
7	One time dip vaccination + One time bath vaccination	1 x dip + 1 x bath

Table 2:

	1 x oral	1 x dip.	2 x oral	2 x dip.	1 x dip. + 1 x oral	1 x dip. + 1 x bath	Percentage survival
Low dose (6.5 x 10^4 cfu/fish)							
Control	NS	NS	NS	***	NS	***	49
1 x oral		NS	NS	***	NS	***	44
1 x dip			NS	NS	NS	NS	69
2 x oral				***	NS	***	44
2 x dip.					NS	NS	97
1 x dip + 1 x oral						NS	68
1 x dip + 1 x bath							92
Medium dose (6.5 x 10^5 cfu/fish)							
Control	NS	**	NS	***	NS	***	11
1 x oral		NS	NS	***	NS	NS	28
1 x dip			***	*	NS	NS	49
2 x oral				***	NS	***	5
2 x dip					***	NS	83
1 x dip + 1 x oral						NS	31
1 x dip + 1 x bath							56
High dose (6.5 x 10^6 cfu/fish)							
Control	NS	NS	NS	**	NS	NS	5
1 x oral		NS	NS	*	NS	NS	13
1 x dip			NS	*	NS	NS	11
2 x oral				**	NS	NS	6
2 x dip					NS	NS	40
1 x dip + 1 x oral						NS	22
1 x dip + 1 x bath							28


A) Low dose (6.5 x 10^4 cfu/fish)
Percent survival
Days post-challenge
Control
1 x oral
1 x dip
2 x oral
2 x dip
1 x dip + 1 x oral
1 x dip + 1 x bath
B) Medium dose (6.5 x 10^5 cfu/fish)
A) High dose (6.5 x 10^6 cfu/fish)


Relative OD450 nm
1.00
0.75
0.50
0.25
0.00
*
*
*
*
*
Control
1 x oral
1 x dip
2 x oral
2 x dip
1 x dip + 1 x oral
1 x dip + 1 x bath
52 days post 1st vaccination
1st sampling
90 days post 1st vaccination
45 days post booster
2nd sampling
119 days post 1st vaccination
74 days post booster
22 days post challenge
3rd sampling

Highlights for the manuscript

- Vaccine induced protection of rainbow trout can be optimized by booster immunization either provided as dip (most effective), bath (less effective) or orally (least effective).
- Oral immunization may be used as booster after dip vaccination but applied as a single oral application it induced merely a slight immune response.
- Oral primary vaccination followed by an oral booster conferred no protection.