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Development and preliminary validation of an antibody filtration-assisted single-dilution chemiluminometric immunoassay for potency testing of *Piscirickettsia salmonis* vaccines

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ABSTRACT

Challenge with live pathogens could be substituted by serology for many veterinary diseases, however little progress has been made in the development of alternative batch vaccine potency tests for fish. This study reports the development and preliminary validation of a single-dilution filtration-assisted chemiluminometric immunoassay (SD FAL-ELISA) applied to measure anti *Piscirickettsia salmonis* IgM in individual or pooled serum and mucus samples. The assay was set up to test a single-dilution of the sample. Serum SD FAL-ELISA yielded a sensitivity of 90% and a specificity of 96%. SD FAL-ELISA was applied to evaluate pooled and individual samples from *P. salmonis* challenge assessments. Relative-light units values (RLU) obtained by SD FAL-ELISA were proportional to antibody levels in serum. RLU values obtained from pooled and individual serum samples increased with the observed relative percent survival (RPS) values, indicating a correlation between protection and specific IgM levels. Results obtained for specific IgM in mucus samples was not related to the RPS, but discriminated the vaccine that yielded high RPS (86.4%) from the others (40.9 and 54.5%). This is the first report on the development of an indirect high-throughput serological assessment for *P. salmonis* vaccine potency testing using both pooled or individual serum and cutaneous mucus samples.

1. Introduction

Salmon Rickettsial Syndrome (SRS), also known as piscirickettsiosis, corresponds to an aggressive infectious disease affecting the world of salmon aquaculture with a notorious economic impact [1–3]. The disease is caused by a non-motile obligate intracellular Gram-negative bacterium, *Piscirickettsia salmonis* (*P. salmonis*) [1,2]. SRS is controlled mainly by the use of antibiotics. There are also inactivated and recombinant vaccines available, though efficacy is variable. New developments are in the pipeline as there is a need of new recombinant or DNA vaccines [4–8].

Evaluation of potency for fish pathogens' vaccines is currently done *in vivo*. Vaccine batch potency testing requires challenge with a virulent strain of *P. salmonis*. Relative Percent Survival (RPS) is calculated by comparison of the survival rate within the control and the test groups at 60% control mortality [9]. This *in vivo* testing is expensive and against current efforts to refine, reduce – and eventually replace – the use of animals for the evaluation of biological products, Russell and Burch's 3*Rs* concept (*R*efine-*R*educe-*R*eplace animals) [10].

Refinement has been defined as "any approach which avoids or minimizes the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their wellbeing" [11]. Serological methods such as specific antibody measurements are considered a potential way of reducing the number of fish routinely being subjected to challenge procedures. However, little progress has been made in the development and validation of such alternative batch potency tests [10].

Two of the major limitations for serological testing in fish, in addition to the usual constraints of serological indirect assessments and their validation and standardization, are the difficulty to obtain

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serum samples from the whole vaccinated group and the little knowledge of correlation between specific IgM and protection against a particular disease. IgM can be measured in serum and has also been detected in salmon cutaneous mucus [12,13]. Small amounts (about 50 μl) of cutaneous mucus can be obtained by gently scratching the skin, without the need of intense handling or trained personnel; reducing costs, manipulation, and animal pain. The limitation of this kind of sample is the concentration factor to yield an amount of IgM that can be measured and the viscosity that interferes with the release of IgM.

We present the development of a **S**ingle-**D**ilution **F**iltration-**A**ssisted chemi**L**uminometric **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay "SD FAL-ELISA" for the evaluation of *P. salmonis* specific IgM in serum and cutaneous mucus of vaccinated salmons. FAL-ELISA [14] allows testing larger sample volumes than regular ELISAs. Samples are filtered through a polyvinyl difluoride (PVDF) membrane coated with *P. salmonis* lysate and specific IgM in the samples is retained. Indirect IgM detection follows and the reaction is finally revealed with luminol/peroxide (chemiluminiscence).

2. Materials and methods

2.1. Vaccines

Ricketvac Oleo® (Recalcine S.A., Santiago, Chile) was used as the whole-bacteria inactivated commercial vaccine, delivered by the intraperitoneal route (IP). The two experimental vaccines corresponds to a saline mixture of *pcDNA™4/HisMax (Invitrogen) plasmids* containing the ORF of *P. salmonis* Hsp60 or Hsp70 [5] (50 μg per fish/intra-muscular route).

2.2. Serum and mucus samples

Mucus and serum samples used to set up the technique were kindly provided by Tecnovax S.A. (Buenos Aires, Argentina). Serum samples were classified as high positive, low positive or negative against *P. salmonis* according to the results obtained in an in-house ELISA (Tecnovax SA). Samples classified as "low positive" were obtained from fish immunized with a low-potency vaccine (15 blood samples, 9 mucus samples); "high positive" ELISA samples corresponded to vaccinated and infected fish that survived the challenge (15 blood samples, 6 mucus samples); fish immunized with a vaccine containing antigens of infectious pancreatic necrosis virus (IPN, 5 serum and mucus samples) and naïve animals (25 serum − 9 mucus samples) were classified as negative samples. High positive, low positive and negative internal control−standards applied in each assay corresponded to pooled samples from these different groups. Other mucus and serum samples from fish immunized with a viral vaccine (IPN), survivors from SRS vaccination/challenge trials and näive animals were also kindly provided by Tecnovax S.A. (Buenos Aires, Argentina).

2.3. Fish immunization and challenge experiments

Atlantic salmon, *Salmo salar*, with an average weight of 30 g were maintained at 13 °C under controlled conditions of *oxygenation* (6.5 ppm), feeding, and water flow in separate tanks (density $\leq$ 15 kg/m^3). A group of 250 fish were divided in 5 groups of 50 animals each and injected with 200 μl of Ricketvac Oleo® (IP) or 100 μl of each experimental DNA vaccine (IM). One group was left untreated and the other received a placebo DNA vaccine (50 μg of empty plasmid per fish in 100 μl volume, IM).

The fish were maintained under controlled conditions in separate tanks. Five days before challenge, performed at 28 days post vaccination (dpv); serum and mucus samples were taken from 15 animals of the treated and control groups under anesthesia [15]. At the same time, five fish per tank were euthanized and sampled to perform necropsy for safety studies. Fish were killed by concussion of the brain by striking of the cranium, as stated in the New EU legislation (Directive 2010/63/EU of the European Parliament and of the Council). Presence of specific IgM against *P. Salmonis* was analyzed by SD FAL-ELISA as stated below.

The challenge was carried out at the Pisciculture Experimental Unit of Tecnovax S.A., located in Mercedes, Buenos Aires, Argentina following standard requirement for registration of commercial vaccines in Chile [16,17]. Infection was conducted by means of intraperitoneal injection of a cultured *P. salmonis* suspension ($\approx 10^8$ TCID-50/ml). *P. salmonis* AG-1 (Chile) was cultured on enriched blood agar following the procedure published by Mauel et al. [18]. Fish were challenged with a dose of 0.1 ml equivalent to 2 times the LD50. LD50 was previously determined by analyzing cumulative mortality of intraperitonally injected fish with serial dilutions of *P. salmonis* Mortality of fish was registered periodically and dead fish were analyzed for lesions. The effectiveness of the vaccine was determined by the relative percent survival 60 [16], which was calculated as follows: RPS = [1 − (% mortality of test group ÷ 60)] × 100%.

2.4. SD FAL-ELISA procedure

The procedure was adapted from the protocol published by Capozzo et al. [14]. Custom-developed PVDF plates were activated with methanol for 25 s. Methanol was removed by filtration and rinsed five times with PBS. SRS-antigen prepared from sonicated bacterial pellets (4 ng/well) was added (in PBS) and left 1 h at room temperature. Coating solution was removed by vacuum and washed twice with 200 μl/well of blocking solution containing BSA in TBS-T, and blocked for 30 min at room temperature. Samples were filtered through a 0.45 μm and a 0.22 μm membrane and dispensed into the wells (by duplicate or triplicate). Volumes over 300 μl were passed through the filter-ELISA plate slow enough as to complete a 15 min incubation time at room temperature. After 4 washing steps with blocking solution, a Mab anti salmon-IgM (Pierce Biotechnology, Rockford, IL USA) was added (dilution 1:1000), incubated for 15 min, washed and revealed with 1:5000 diluted anti-mouse IgG + IgM (H + L) conjugated to peroxidase (HRP) (Jackson ImmunoResearch, West Grove, PA USA). The reaction proceeded with the addition of Chemiluminescent Peroxidase Substrate (Sigma). Following 5 min incubation, plates were read using a photo-luminometer (1 s integration time). Read-outs were given in Relative-Light Units (RLU). Data was transferred to an Excel sheet for further analysis. Each plate was validated relative to signal to noise (NIL) values of duplicate mean RLU of the control samples. Corrected RLU values were obtained by the ratio between RLU readings for the sample and the NIL control (signal to noise ratio). Results were expressed as the Mean of corrected Relative-Light Units cRLU ± SD.

Serum samples were titrated for the set up (starting 1:10) and finally used in a single 1:200 dilution (5 μl of sample, 1 ml final volume). Mucus samples were tested in serial dilutions from 1:5 to 1:5000. Optimal dilution was 1:50. Dilutions were performed in 2 ml of PBS-T buffer and incubated for 15 min at 42 °C with gentle agitation to release IgM from mucus and avoid filter clogging. The whole volume was filtered through each well.

2.5. Statistical analysis

Statistical analyses were carried out using biostatistics, curve fitting and scientific graphing softwares (GraphPad Prism v4.02. GraphPad Software. USA; MedCalc v11 Software. MedCalc.

Belgium). Significance was established within the 95% confidence interval for all determinations.

Positive (high and low) and negative control samples were run in triplicate 5 independent assays. Intra-assay, inter-assay and replicates' variations were calculated by ANOVA for a nested model of variance, applied to quantify the interaction between repeatability and intermediate precision. Receiver operating characteristic (ROC) analysis, following Delong's method [19] was applied to calculate the preliminary cut off value for SD FAL-ELISAs. The variation coefficient was computed as the "standard deviation/mean" ratio (in percentage). Intra plate variations were calculated for each plate and averaged. Positive and negative samples were compared using *T*-test.

3. Results

3.1. Set up of SD FAL-ELISA to measure SRS-specific IgM

Initial set up experiments followed the steps outlined in our previous publication [14]. Several concentrations of bacterial lysate were evaluated for coating the PVDF plates, ranging from 20 to 1 ng per well. The assay performed equally when using 10 and up to 3 ng of bacterial lysate per well. A concentration of 4 ng was selected, which allows having a cost-effective yield without affecting the assay's performance.

In order to determine the sample-working range, positive control (pooled sera from infected animals), low positive control (from vaccinated fish) and negative pooled sera were tested in serial dilutions from 1:10 to 1:2500 in FAL-ELISA, following the standardized protocol. Sera from salmons vaccinated with a viral vaccine were also included (IPN vaccinated curve, Fig. 1). Samples were tested individually, in three independent assays. Fig. 1 shows the dilution curves of control sera and mucus samples in one representative assay. FAL-ELISA applied to serum (Fig. 1A) could clearly differentiate between high positive and negative samples at all dilutions. It was also possible to discriminate between the strong positive, the low positive and the negative sample at a dilution of 1:200. This dilution was selected to assess sera in the single-dilution assay. Variation coefficients for positive and negative controls (pooled samples) at a 1:200 dilution were found to be CV = 19% and 15% between replicates and CV = 19% and 20% between assays; respectively.

FAL-ELISA set up for mucus samples was performed equally. In order to enhance sensitivity, samples were diluted in 2 ml and heated before testing, and the whole volume was applied to each well. Background RLU values (mucus from naïve and IPN-vaccinated fish) were higher with mucus than serum samples and detection limits, lower. Attempts to reduce background with different antigen coating concentrations, blocking times and blocking solutions were not successful. FAL-ELISA applied to mucus could not differentiate low positive from negative samples (Fig. 1B). However, there was no certainty about the presence of IgM in these low positive mucus samples, as in-house ELISA assessments were negative, probably due to low sensitivity. Even total IgM was undetectable by a standard western blot analysis [20] (data not shown).

Mucus samples obtained from fish with high levels of serum SRS-specific IgM were negative in the in-house mucus-ELISA but total IgM was detectable by western blot. These mucus samples were discriminated from those obtained from naïve fish or from IPN vaccinated salmons at a 1:50 dilution in FAL-ELISA. In this conditions variability between measurements were similar to those found for serum samples (<20%).

3.2. Ability of SD FAL-ELISA to discriminate between positive and negative samples

In order to fix a preliminary cut off value, 30 sera from naive salmon or from salmon vaccinated with a viral IPN-vaccine were evaluated. Positive samples were from vaccinated (commercial vaccine trial) and challenged animals. Sera were diluted 1:200 and tested by SRS SD FAL-ELISA. Mucus samples (9 from naive and 15 from vaccinated fish) were assessed in a 1:50 dilution as stated above.

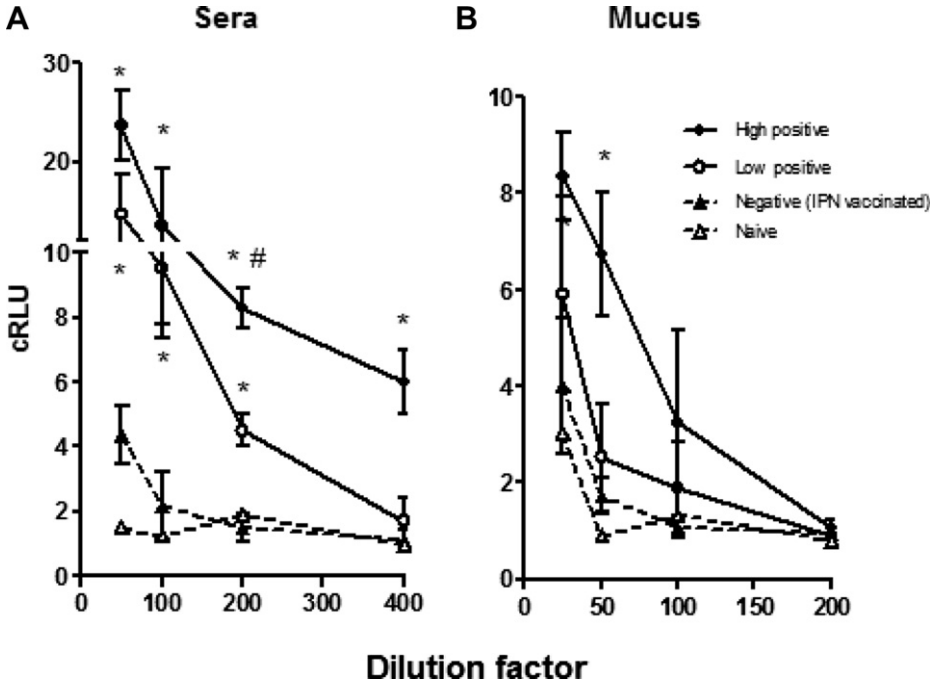

Fig. 1. *Determination of sample-dilution working range.* FAL-ELISA was applied to serial dilutions of control sera or mucus samples (A and B, respectively). The curves correspond to triplicate determinations in a representative assay. Mean cRLU ± SD values for each dilution are depicted. *Significantly higher than negative control and naïve fish ($p < 0.05$). #Significantly higher than the low positive control cRLU value ($p < 0.05$).

Differences between cRLU values obtained from positive or negative samples were significant for serum samples ($p < 0.05$, Fig. 2A). A preliminary estimate of the assay's cut off value was calculated by using an ROC analysis. ROC curve for serum samples is depicted in Fig. 2B. When using a cut off value of cRLU = 2.4; the assay was 90% sensitive and 96% specific.

SD FAL-ELISA did not detect specific IgM in mucus from those fish with low serum IgM levels. cRLU values were close to negative measurements (gray circles, Fig. 2C). IgM in these mucus samples could not be detected with other methods (in-house ELISA and Western blot, data not shown). Consequently, these samples were considered as "negative" for the analysis. In this condition, sensitivity and specificity were estimated. Cut off value for mucus samples was fixed at cRLU = 2.4, the same than for serum samples; which renders 90% sensitivity and specificity (ROC curve, Fig. 2D).

3.3. Assessment of SD FAL-ELISA on individual and pooled serum samples in a vaccine trial

A challenge experiment was performed to evaluate the association between protection against *P. salmonis* and specific IgM measured by the SD FAL-ELISA. Groups of 50 fish/each were vaccinated with a commercial vaccine or two experimental vaccines. Serum and mucus samples were taken from 15 animals and evaluated individually and in three pools of 5 samples each by SD FAL-ELISA (Fig. 3). Animals were challenged five days later and RPS values recorded. RPS values were of 40.9% and 54.5% for the experimental vaccines while the whole-inactivated commercial vaccine was fully protective (RPS = 86.4%). Fish immunized with empty plasmid were not protected (RPS = 13.6%).

Evaluation of individual and pooled samples rendered similar results than mean cRLU from individual samples. When using serum samples, cRLU values were clearly related to RPS values. This could be observed either testing individual or pooled samples (Fig. 3A and B, respectively). Based on these results we built a correlation curve (Fig. 4). Correlation coefficient between RPS and cRLU was 96.4%, and followed a linear regression.

The evaluation of mucus by SD FAL-ELISA also showed that either pooled or individual samples gave similar results (Fig. 3C–D). Though RPS and cRLU values from mucus samples did not correlate, when applying the cut off value to discriminate positive and negative samples, mucus-IgM levels induced by the commercial vaccine were rated as positive samples. Thus, cRLU values obtained from individual or pooled mucus samples may be applied to assess the outcome of a commercial vaccine potency test, as only vaccines able to induce high RPS values would be released to the market.

4. Discussion

Potency assessments for fish vaccines are currently performed *in vivo* [6]. Though many serology-based studies have been developed against various fish diseases [13,21,22] only a few attempts have been made recently to study the feasibility of replacing challenge with *in vitro* assessments [23,24]. In this manuscript we described the development of a SD-FAL-ELISA that measures specific anti *P. salmonis* IgM in serum and cutaneous mucus samples, and performed a preliminary validation applying the assay in a challenge experiment.

P. Salmonis IgM-FAL-ELISA has been set up as a single-dilution assay that uses 5 μl of serum and 50 μl of cutaneous mucus. These amounts of sample can be easily obtained. Sampling 50 μl of mucus can be done directly with a vial thus minimizing animal manipulation, while obtaining larger amounts may be impractical or stressful for the fish.

Cutaneous mucus is a stable environment for IgM. Hatten et al. [20] demonstrated that IgM in cutaneous mucus did not result in visible degradation, even after 17 h at 4 °C. However, low concentration of IgM in mucus is a major issue for a serological assessment. We have found that SD FAL-ELISA can detect up to 5 pg of immunoglobulins per well when target antigen is bound to the PVDF membrane (unpublished data). Even though more assays are needed to calculate the detection limit of this particular assay, considering that rough estimates of the amounts of IgM in mucus were below 15 μg IgM ml^{-1}; our assay that uses 50 μl of mucus

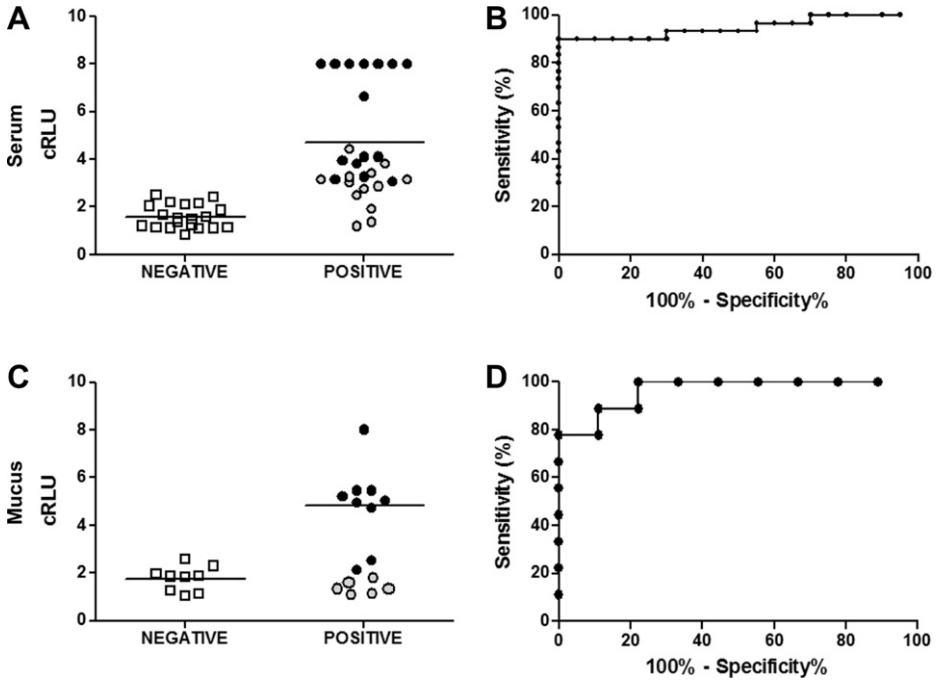

Fig. 2. *Assessment of SD FAL-ELISA cut off value*. cRLU values obtained for positive and negative serum (A) and mucus (C) samples. Panels B and D depict ROC curves for the established cut off cRLU value (2.4) of serum and mucus samples, respectively. Gray circles: low positive samples; black circles: high positive samples.

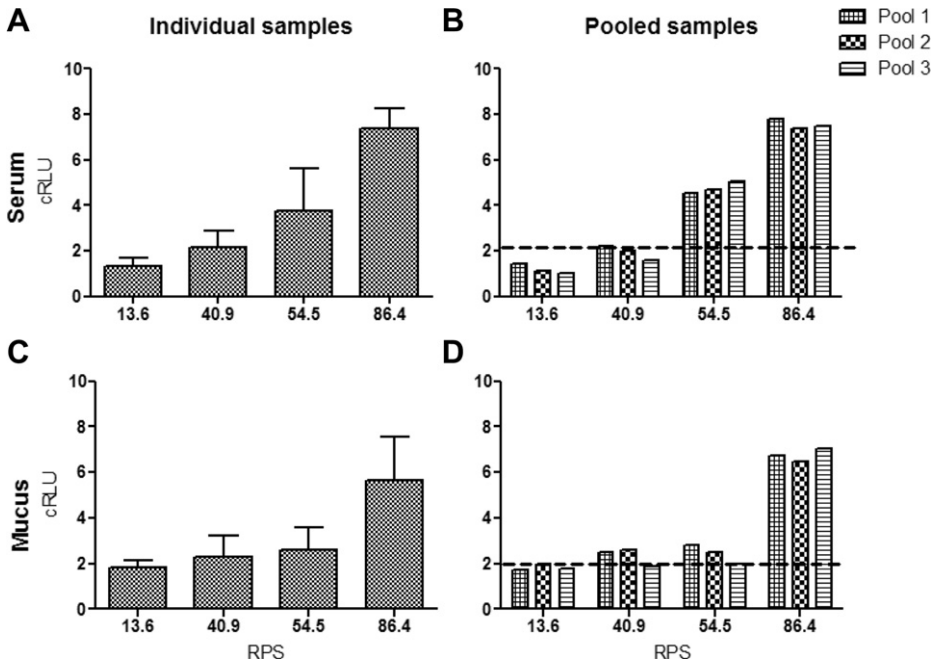

Fig. 3. *Performance of SD FAL-ELISA in a vaccine trial.* (A–C) Mean + SD cRLU values of 15 individual samples per group related to the RPS values obtained in a challenge trial, assessed in serum or mucus samples, as indicated. (B–D) cRLU values obtained with three serum or mucus pooled samples (5 samples each) for each vaccine, regarding their RPS values. The dotted line depicts the cut-off of the assay.

(containing about 750 pg of total IgM) may be able to detect specific IgM if present, at least, as 0.67% of the total amount. The assay can also be modified to increase the initial amount of mucus thus enhancing sensitivity. Higher amounts of sample can be applied as FAL-ELISA is not limited in sample-volume like regular ELISAs.

SD FAL-ELISA detected specific IgM in mucus from fish with high IgM serum levels, but not in those with low-IgM serum titers. We are not certain about the actual presence of IgM in mucus of those fish with low serum IgM levels. A relationship between IgM levels in serum and mucus has been reported in fish [25]. However, some vaccines induced high IgM serum levels with low or undetectable antibodies in mucus [12,13]. Nevertheless, the capacity of SD FAL-ELISA of identifying high responders, which are related with high RPS values, can be enough to accept or reject a commercial vaccine batch.

SD FAL-ELISA specific IgM assessment in serum was more sensitive than in mucus samples, allowing the discrimination between low and high positive samples. Interestingly, Relative-light units values (RLU) obtained by SD FAL-ELISA were proportional to antibody levels and related to the RPS, indicating a correlation between the levels of specific IgM in serum and the protective capacity of a vaccine. A positive correlation between survival and the levels of specific IgM have been demonstrated for other fish diseases [21,26] but had never been described for *P. salmonis*. However, presence of higher levels of specific IgM have been related to multi-vaccination with high payload SRS vaccines [27] which rendered high RPS values in challenge experiments.

This is the first report that demonstrates a correlation between serum IgM levels and RPS against *P. salmonis* and on the development of an indirect serological evaluation for SRS vaccine potency testing. The assessment of the samples in single-dilution and the possibility of testing pooled samples optimize costs and simplify the procedure. The technology can suit vaccine development, that need sensitive tests able to differentiate vaccines of different potency, and commercial applications that may only require to establish a cut off value that ensures the presence of high levels of

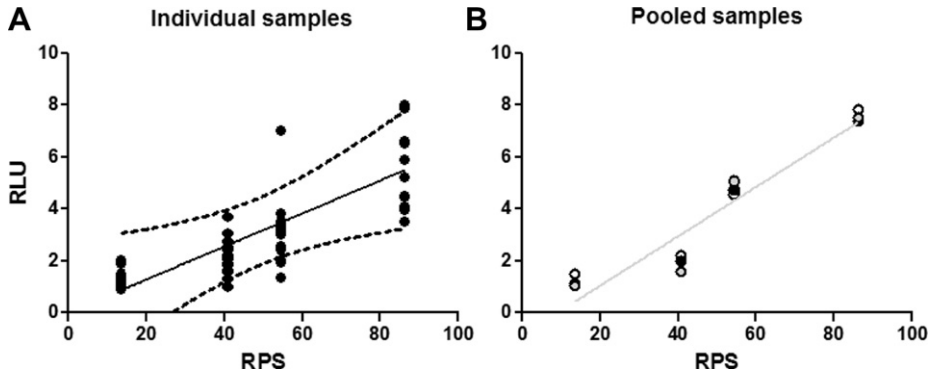

Fig. 4. *Correlation between cRLU and RPS values*. Correlation between results obtained by SD FAL-ELISA (cRLU values) and protection (measured as RPS values) was computed for individually tested serum samples (A). Linear regression curves and intervals of 95% prediction band are indicated with full and dotted lines, respectively. Each dot corresponds to an individual value. (B) Correlation assessment for pooled samples. Each circle depicts cRLU value for a pool of 5 serum-samples.

specific IgM, related to high RPS. For this application, mucus SD FAL-ELISA from pooled samples can be an interesting low-cost alternative to explore. Further studies conducted with vaccine batches comprising different antigen payload are needed to validate the high-throughput application of this assay.

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