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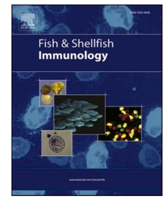

Full length article

Detection of specific Atlantic salmon antibodies against salmonid alphavirus using a bead-based immunoassay

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ABSTRACT

Salmonid alphavirus (SAV) is the etiological cause of pancreas disease (PD) in Atlantic salmon (*Salmo salar*). Several vaccines against SAV are in use, but PD still cause significant mortality and concern in European aquaculture, raising the need for optimal tools to monitor SAV immunity. To monitor and control the distribution of PD in Norway, all salmonid farms are regularly screened for SAV by RT-qPCR. While the direct detection of SAV is helpful in the early stages of infection, serological methods could bring additional information on acquired SAV immunity in the later stages. Traditionally, SAV antibodies are monitored in neutralization assays, but they are time-consuming and cumbersome, thus alternative assays are warranted. Enzyme-linked immunosorbent assays (ELISAs) have not yet been successfully used for anti-SAV antibody detection in aquaculture. We aimed to develop a bead-based immunoassay for SAV-specific antibodies. By using detergent-treated SAV particles as antigens, we detected SAV-specific antibodies in plasma collected from both a SAV challenge trial and a field outbreak of PD. Increased levels of SAV-specific antibodies were seen after most fish had become negative for viral RNA. The bead-based assay is time saving compared to virus neutralization assays, and suitable for non-lethal testing due to low sample size requirements. We conclude that the bead-based immunoassay for SAV antibody detection is a promising diagnostic tool to complement SAV screening in aquaculture.

1. Introduction

Salmonid alphavirus (SAV), also named *Salmon pancreas disease virus* (SPDV) by the International Committee on Taxonomy of Viruses, is a widespread pathogen in European aquaculture, causing pancreas disease (PD) and sleeping disease (SD) in Atlantic salmon (*Salmo salar)* and rainbow trout (*Oncorhynchus mykiss*) respectively. The diseases are widespread in Europe and may cause high fish mortality and reduced weight gain resulting in decreased fish welfare and great economic losses for the industry. The six subtypes of SAV (SAV1-6) are geographically separated: SAV1 is common in Ireland and Scotland, SAV2 in rainbow trout reared in freshwater in continental Europe [1] as well as in salmonids in sea cages in England, Scotland and Norway [2], and SAV3 is common in Norway, where the disease has been recognized since 1989 [3]. SAV4, 5 and 6 are occasionally detected in the British Isles [4]. The subtype classification is based on the nucleotide sequence encoding the SAV proteins nsP3 and E2 [5]. There is a general cross-neutralization between antibodies produced against all subtypes except SAV6, which represents a different serotype [6]. SAV-infected fish demonstrate reduced appetite, aberrant swimming, fecal casts and lethargy, and the population has increased mortality. Subclinical infection can also occur [7]. Histopathological changes, seen as degeneration and necrosis, are present in pancreas, heart and skeletal muscle. The main route of SAV transmission is horizontal, through virus shed in water. The virus can spread between farms via water currents or moving

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Abbreviations

µ1c	Capsid protein µ1c from PRV virus
CHH	Chum Salmon Heart cells
CHSE	Chinook Salmon Embryo cells
DNP-KLH	2,4-Dinitrophenyl-Keyhole Limpet Hemocyanin (hapten-carrier complex)
FP	antigen from Infectious Salmon Anemia Virus
GCN	Genome Copy Number
HSMI	Heart and Skeletal Muscle Inflammation
i.p.	Intraperitoneally
ICP11	antigen from White Spot Disease Virus in shrimps
Ig	Immunoglobulin
LM	Lipid Modified
MFI	Median Fluorescence Intensity
NA	(Virus) Neutralization Assay
nsP	Non-Structural Protein
OIE	World Organisation for Animal Health
ORF	Open Reading Frame
PD	Pancreas Disease
PRV	Piscine Orthoreovirus
RC	Replication Complex
SAV	Salmonid Alphavirus
SAV-E1/E2	Glycoproteins E1 or E2 from SAV virus
SAV-TX	Triton-X treated SAV particles
$TCID_{50}$	Fifty Percent Tissue Culture Infective Dose
WPC	Weeks Post Challenge
xMAP	Multi-Analyte Profiling (™ Luminex Corp)

of infected fish, unless proper biosafety measures are implemented [8–10].

SAV is an enveloped virus with a capped, polyadenylated, single-stranded RNA genome with positive polarity. The genome is 11.9 kb and consists of two large open reading frames (ORFs). The ORF1 encodes four non-structural proteins (nsP1-4), and the ORF2 encodes five structural proteins; the capsid, E3, E2, 6K and E1 proteins. The virion consists of an icosahedral nucleocapsid that encloses the genome, and a surrounding lipid envelope derived from the host cell [11]. The major spikes embedded in the lipid envelope consist of trimers of E2 and E1 glycoprotein heterodimers. Both E1 and E2 are vital for viral entry into new cells, with E2 being responsible for receptor recognition and binding, and E1 involved in host cell membrane penetration. During SAV replication, the non-structural region is translated from the viral genomic RNA into a replication complex (RC). The RC replicates the viral genome and transcribes the second ORF, which is translated into the structural polyprotein capsid-p62-6K-E1. The capsid is cleaved from the structural polyprotein and the remaining p62-6K-E1 translocate to the endoplasmatic reticulum, where E1 and E2 undergo post-translational modifications prior to budding [12].

According to the OIE Manual of Diagnostic Tests for Aquatic Animals 2019, a suspected case of infection with SAV is defined as the presence of one of the following: Clinical signs, gross or microscopic pathologic lesions consistent with the disease, detection of SAV, detection of antibodies against SAV, or known contact with infected or suspected cases. The presence of SAV is confirmed when at least two independent laboratory tests (histopathology, cell culture, RT-qPCR or virus neutralization assays) applied on the same individual fish are positive. RT-qPCR is well suited for detection of virus in early infection and during clinical disease, and currently, all marine production sites in Norway sample hearts from 20 fish monthly for RT-qPCR testing [13]. To be at least 95% sure to detect at least one SAV-positive fish in 20 random samples, the prevalence or infection rate must be at least 15%.

While serological methods are widely used in terrestrial animals for diagnosis, vaccine efficacy studies, surveillance, and the demonstration of freedom from disease, the use of such methods have been more limited in aquaculture [14]. RT-qPCR will usually be more sensitive at peak virus infection, but measurements of serum antibody responses may have a diagnostic advantage in the later period of an infection, in populations with subclinical infection and low prevalence of infected fish, and in cases where SAV RT-qPCR results are on the borderline between positive and negative. Neutralizing antibodies are detected in SAV infected fish from around two weeks after infection [15,16]. In field trials, neutralizing antibodies against SAV have been shown to persist in a fish population for as long as 14 months [17]. Virus neutralization assays are based on the ability of plasma or serum to inhibit virus replication in cell culture after pre-treatment of virus with the samples of interest, and are in use for routine diagnostics and to study epidemiology in the United Kingdom and Ireland [18–21]. A longitudinal study of two outbreaks of PD caused by SAV1, indicated that a neutralization test was more sensitive and could detect fish with antibodies against SAV for a longer period than RT-qPCR could detect virus [17]. Positive neutralization tests have also been found at slaughter from fish from farms deemed free of SAV through the whole sea water phase by the monthly RT-qPCR testing in Norway [22]. However, virus neutralization assays require personnel experienced with viral culturing and is time consuming, as the incubation time for virus growth in cell culture is at least three days [19]. An immunohistochemical method for SAV detection has been published [23], but could only detect virus in samples from the acute stages. Enzyme-linked immunosorbent assays (ELISAs) using recombinant E1 or E2 proteins as antigens have been used in research to detect antibodies against SAV [24,25], but have not been in use for field diagnostics.

Bead-based immunoassays, such as the xMAP® assay, can be used to simultaneously detect antibodies against several pathogens, and we have previously used the method to detect antibodies against *Piscine orthoreovirus* (PRV) [26,27]. Such assays have been used to detect antibodies in mammals for almost two decades [28,29]. The analysis can be done in a few hours and requires only a few microliters of sample material, making the assay suitable to combine with non-lethal sampling. As high background binding and high levels of non-specific antibodies are found in plasma from Atlantic salmon, a functional serological assay requires antigens that primarily bind specific antibodies. We have here tested the xMAP method with a bacterially expressed recombinant SAV3 E2 protein, whole SAV3 particles and disrupted SAV3 particles as antigens. The method was tested on Atlantic salmon plasma from an experimental SAV2 and SAV3 infection study and field samples from a PD outbreak caused by SAV2. We also compared the method with a virus neutralization assay, and with RT-qPCR.

2. Materials and methods

2.1. Cells

Chinook salmon embryo cells, (CHSE-214, hereafter referred to as CHSE) (RRID:CVCL_2780), were grown at 20 °C in Leibovitz (L15) medium supplemented with 10% heat inactivated fetal bovine serum (FBS), L-glutamine (2 mM), 2-mercaptoethanol (40 µM) and gentamicin-sulphate (50 µg/ml) (all from Life technologies, Paisley, Scotland, UK).

A transgenic CHSE cell line expressing structural polyprotein of SAV1 was generated as follows. The coding sequence for the full polyprotein from SAV1 isolate 4640 [30] was synthesised by GeneArt® gene synthesis service (Thermo Fisher Scientific). Three restriction sites (*Nhe*I, *Mlu*I and *Xho*I) present in the coding sequence of SAV1-4640 were removed by incorporating single nucleotide silent modifications, using

the salmon codon usage, in the sequence data supplied to the DNA synthesis company. For the purpose of sub-cloning, *Mlu*I, *Xho*I and *Hind*III, *Nhe*I and *Sal*I sites were also added in the synthesised sequence, upstream and downstream of the ORF respectively. The resulting 3945 nucleotide pp4640 coding sequence was sub-cloned into the pcDNA3.1-Hyg-mEGFP [31] vector between the sites *Xho*I and *Hind*III to generate pcDNA3.1-Hyg-mEGFP-pp4640 (ppG). Stable CHSE-ppG transfected CHSE cell lines were generated as described previously [31]. CHSE-ppG cells were selected and maintained in CHSE culture medium supplemented with 30 μg/mL Hygromycin (Invitrogen).

2.2. Virus propagation

CHSE-ppG cells were used to propagate the SAV3 H20/03 strain (acc. no. DQ149204). The cells were grown in 162 cm^2 Costar flasks (Corning Incorporated, Corning, NY, USA) for 48 h prior to infection. The medium was removed, and cells washed twice with PBS before adding 5 ml virus suspension (3×10^7 $TCID_{50}$/ml) diluted 1:1000 in non-supplemented L15 medium and incubated for 1 h at 15 °C. This was followed by addition of 35 ml L-15 medium supplemented with 2%FBS, 2-mercaptoethanol (40 μM) and hygromycin (30 μg/ml) and continued incubation at 15 °C for 10 days. Flasks were then transferred directly to −80 °C.

2.3. Concentration of SAV particles

SAV particles were concentrated by pelleting, using ultracentrifugation following a protocol developed for the alphavirus Semliki forest virus [32], with modifications. The samples were kept on ice throughout the entire protocol and cell lysis was performed using the freeze-thaw method on ten 162 cm^2 flasks containing infected CHSE-ppG and non-infected CHSE cells. The lysed cell suspensions were then transferred to 50 ml centrifuge tubes (Corning Incorporated, Corning, NY, USA) and centrifuged at 10,000 g for 20 min at 4 °C. The supernatants were transferred to five 94 ml polyallomer centrifuge tubes, and the tubes sealed with aluminum caps (Beckman-Coulter, Brea, CA, USA). Ultracentrifugation was performed at 10,00,00 g for 150 min at 4 °C using a fixed angle Beckman Type 45 TI rotor in an Optima LE 80 K Ultracentrifuge (Beckman-Coulter, Brea, CA, USA). The top aqueous phase was removed, and the pellets suspended in 200 μl PBS and incubated overnight at 4 °C with 250 rpm rotation. The suspensions were pooled together and were then stored at 4 °C until further use.

2.4. RNA extraction and RT-qPCR

Quantitative real-time PCR was performed to confirm the presence of virus and to measure the genome copy number (GCN) ml^{-1} in the pooled pellets. Extraction of viral RNA was performed using the QIAamp viral RNA mini kit (Qiagen, Venlo, Netherlands) following the protocol of the manufacturer. Elution of RNA was performed with a 40 μl volume of elution buffer from the same kit. The RT-qPCR was performed using the Qiagen One-step RT-PCR kit and 3 μl eluted RNA in each reaction. Primers and probe, targeting a highly conserved region of the SAV nsp1 coding sequence, have previously been described [33]. The conditions used in each reaction were 400 nM of each primer, 300 nM probe, 0.5 μl dNTP mix, 0.32 MgCl, 2.5 μl OneStep RT-PCR buffer, 0.5 μl OneStep enzyme mix and 2.3 μl RNase-free water in a total volume of 12.5 μl. For the generation of a SAV standard curve, 10-fold dilutions of a plasmid containing the complete SAV3 genome, the lowest concentration representing one plasmid copy and highest 10^9 copies, were run in triplicate together with the viral samples. The cycling parameters used were 50 °C/30 min and 95 °C/15 min, followed by 40 cycles of 95 °C/15 s, 60 °C/1 min on an AriaMx (Agilent, Santa Clara, CA, USA). The presence of SAV in the pellet was confirmed with a C_t of 7.5. The standard curve slope, regression coefficient and amplification efficiency were −3.45, 0.999 and 94.3, respectively (Suppl. Fig. 1). This indicates that the standard curve was near optimal and can be used for absolute quantitation. By extrapolation we get a GCN of 1.4×10^{11} ml^{-1}.

2.5. End-point dilution assay

The titer of infectious virus per ml ($TCID_{50}$ ml^{-1}) in the pelleted virus fraction was determined by end-point dilution assay in chum salmon heart-1 cells (CHH-1) (RRID:CVCL_4143). Serial 10-fold dilutions were made of viral pellet suspension, and 50 μl of each dilution was added in eight parallels onto a 96-well plate containing a confluent monolayer. The infection protocol was as described above. The plates were incubated for ten days for the appearance of cytopathic effects. CHH-1 cells were fixated and stained using an intracellular Fixation and Permeabilization buffer (eBioscience, San Diego, CA, USA) and washed in Dulbecco's PBS (DPBS) with sodium azide. Cells were incubated with 17H23 anti-SAV E2 primary mouse antibody [34] followed by Alexa Fluor® 488 anti-mouse secondary antibody from Molecular Probes (Life Technologies). Dilutions of primary (1:1000) and secondary (1:400) antibodies were performed with permeabilization buffer in accordance with the manufacturer's protocol. For nuclear staining, Hoechst trihydrochloride trihydrate (Life Technologies) was used. The number of positive wells was quantified using an Olympus IX81 inverted fluorescence microscope. The Spearman-Kärber algorithm was used for calculation of $TCID_{50}$ ml^{-1}. The pelleted virus suspension contained 2.0×10^{10} $TCID_{50}$ ml^{-1}.

2.6. SAV-E2 antigen

The SAV E2 protein was custom made by the MRC PPU Reagents and Services, University of Dundee (https://mrcppureagents.dundee.ac.uk) using the following procedure. The full-length E2 coding sequence (acc. no. JQ799139), presented in the plasmid pGex-SAV3 E2, was expressed as a GST-fusion protein in *E. coli* (BL21) by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) (50 μM). Bacteria were cultured for 16 h before harvesting by centrifugation at 4200×*g* for 20 min at 4 °C. The pellet was resuspended in ice cold *E. coli* lysis buffer (50 mM Tris/HCl pH7.5, 250 mM NaCl, 1% Triton-X, 0.1% 2-mercaptoethanol, 1 mM Pefabloc (4-(2-aminoethyl)-benzene-sulfonyl fluoride) and 1 mM benzamidine). Bacteria were lysed using a cell disruptor and extracts clarified by centrifugation at 30 000×*g* for 20 min at 4 °C. Proteins were then purified using Glutathione S-transferase Agarose using standard procedure. Upon elution of the protein with elution buffer (wash buffer + 20 mM glutathione pH 7.5), the fractions containing protein were pooled with end over end mixing. This was subjected to purification on a HiTrap Heparin Sepharose HP column (GE Healthcare) equilibrated in equilibration buffer (50 mM Tris/HCl pH7.5, 0.03% (v/v) Brij 35, 0.1 mM EGTA, 0.1% 2-mercaptoethanol). A linear salt gradient with increasing concentration up to 1 M NaCl was applied to the column and SAV E2 containing fractions were pooled and dialyzed into storage buffer (50 mM Tris/HCl pH7.5, 150 mM NaCl, 270 mM sucrose, 0.03% (v/v) Brij 35, 0.1 mM EGTA, 0.1% 2-mercaptoethanol). The pooled protein was incubated for 16 h with GST-PreScission Protease at 4 °C and a heparin column was used to separate the cleaved GST, GST-PreScission Protease and the SAV E2. The expression and protein yield were analyzed by SDS PAGE and Coomassie Blue staining.

2.7. Experimental infection, sample collection and plasma samples

Plasma from seawater-adapted Atlantic salmon post-smolts was collected in a previously published SAV-PRV co-challenge trial [35,36]. Only plasma from the SAV-infected control group from the former study (not co-infected with PRV) was used in the present work. The fish had been challenged as described [35]; briefly, the fish were kept in filtered and UV-radiated seawater (34‰ salinity), 12 °C (±1 °C) and on 24 h light. After two weeks, shedder fish were i.p. injected with SAV2 or SAV3 (cell culture medium at a concentration of 10^4 $TCID_{50}$/ml) and introduced to naïve fish four days later. Six cohabitant fish from each

SAV subtype infection were sampled at each sampling point (1, 2, 3 and 6 weeks post challenge (wpc)). Blood was collected from the caudal vein on heparinized vacutainer tubes. After centrifugation, plasma was collected and stored at −80 °C. Much of the plasma from 6 wpc had been used for other purposes, and only two samples from each subtype were available for analysis with the xMAP assay.

As a control for non-specific binding, plasma from a PRV-1 trial described in Lund and co-workers [37] was used. These samples had previously been used to detect antibodies against PRV [27].

The trials were approved by the Norwegian Animal Research Authority and performed in accordance with the recommendations of the current animal welfare regulations as outlined in: FOR-1996-01-15-23 (Norway).

2.8. Field plasma samples

The field samples originated from a clinical PD outbreak caused by SAV2 in an endemic area (Southern Trøndelag), as described in Røsæg and co-workers (pen number 10) [38]. In short, the sampled fish were 1-year-old smolt of AquaGen strain, vaccinated with Alpha Ject micro 6 by Pharmaq and sea transferred in July 2014. During the first fall, the site was diagnosed with heart and skeletal muscle inflammation (HSMI), and the first detection of SAV-RNA from surveillance samples at site level was in April 2015. The fish group was then sampled regularly at 2-3-week intervals from the first SAV-RNA detection until slaughter. Clinical PD was first reported in week 27 in 2015 (July 29, 2015), based on evaluation from the fish health service, the site manager and a coinciding drop in appetite. This was denoted as week 0 of PD. At week 0 the average fish weight was 2.1 kg, and the cumulative mortality was 10.7%. At slaughter, the cumulative mortality was 14.35% and the average slaughter weight was 4.2 kg.

The samples included in this study were composed of nine sampling points from 3 weeks prior to 15 weeks post the onset of clinical PD. Blood samples from ten fish per sampling point were collected in EDTA vacuum tubes, kept refrigerated and centrifuged within 24 h. Plasma was collected and stored at −20 °C. For detection of SAV-RNA, the heart apex was sampled, stabilized in RNAlater® and either held refrigerated for 36–48 h before an overnight shipment or frozen below −20 °C prior to shipment. The analyses were performed by PatoGen AS according to validated and accredited methods (ISO17025 standard). Samples with no detection of virus were assigned a Ct of 38 (cut-off value) to include them in the graphical presentation.

2.9. Antigen preparation and coating on beads

Three different mixtures with whole virus were made from virus suspension from CHSE-ppG cells and coated on beads: 1) diluted 1:1 with PBS (SAV1:1), 2) diluted 1:10 with PBS (SAV1:10), and 3) mixed 2:1 with 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) in PBS and kept for 1 h at room temperature before coating (SAV-TX). Untransfected and uninfected CHSE cells were used as a background control, given that uninfected ppG will still express SAV1 structural protein antigens. These were prepared by mixing two parts PBS containing the pellet from CHSE cells with one part PBS containing 0.1% Triton X-100 and kept for 1 h at room temperature before coating on beads (CHSE control). 60 μl of recombinant E2 protein from SAV3 (0.2 mg/ml) was used per coupling reaction for beads with E2 (SAV-E2). All coating was done according to the producer's instructions and as previously described [26,27] using MagPlex® Microspheres (beads) #12, #21, #26, #29, # 34, #46 and #64 (Luminex Corp., Austin, TX, USA), N-Hydroxysulfosuccinimide sodium salt (Sigma-Aldrich), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Sigma-Aldrich) and Bio-Plex Amine Coupling Kit (Bio-Rad, Hercules, CA, USA). PRV μ1c and DNP-KLH, a hapten-carrier antigen often used to detect non-specific antibodies [39,40], were conjugated to the beads as described earlier [26,39].

2.10. xMAP assay

Titrations of plasma was performed, and a 1:50 dilution was found to give the best discrimination between control fish and infected fish from an experimental challenge trial (data not shown). This dilution was used for all samples except for when SAV-E2 and DNP-KLH beads were analyzed. Here, a 1:400 dilution was used to stay below the upper limit of quantification. As previously described [27], plasma was heated at 48 °C for 20 min to minimize non-specific antibody binding. The immunoassay was performed as described earlier [26]. In short, plasma was diluted 1:50 or 1:400 in PBS and incubated at room temperature for 30 min on a shaker with the coated beads in a Bio-Plex Pro™ Flat Bottom Plate (Bio-Rad). Each sample was incubated with mouse Anti-Salmonid-immunoglobulin (Ig) monoclonal antibody (clone IPA5F12; Cedarlane, Burlington, Ontario, Canada) (raised against salmonid Ig heavy chain, shown to correspond with both identified isotypes of Atlantic salmon IgM [41]) diluted 1:400, biotinylated goat AntiMouse IgG2a antibody (Southern Biotechnology Association, Birmingham, AL, USA) diluted 1:1000 and finally Streptavidin-PE (Invitrogen, Carlsbad, CA, USA) diluted 1:50. Each incubation was performed at room temperature on a shaker at 600 rpm for 30 min. Three washings were done between each incubation. Control beads were coated with proteins from white spot disease virus (ICP11), and from infectious salmon anemia virus (FP native and FP-LM), previously described [27]. Samples were read using a Bio-Plex 200 (Bio-Rad), with median fluorescence intensity (MFI) as quantitative readout. The results were analyzed with the Bio-Plex Manager 6.1 (Bio-Rad). The DD-gate was set to 5000–25000, and between 55 and 208 beads from each bead number were read from each well. The reading was carried out using a low PMT target value.

2.11. Neutralization assay

Samples from the field PD outbreak were heat-treated (48 °C for 20 min) and a virus neutralization assay was performed as described earlier [19,36] with CHSE cells and acetone fixation before antigen detection with immunofluorescence. Endpoint titration of samples was performed if virus neutralization was observed at a 1:20 dilution.

2.12. Statistics

Statistical analyses used were the Mann-Whitney test to detect significant differences in binding to beads, linear regression to examine the correlation between MFI from the different beads and Spearman's correlation to assess the relationship between MFI from beads and neutralization data (GraphPad Prism 7 and JMP Pro 14).

3. Results

3.1. Virus quantification

CHSE-ppG cells infected with SAV-3 H20/03 yielded a viral titer of 3.4×10^9 $TCID_{50}$/mL, considered high compared to CHSE cells, which typically produce a titer of approximately 10^6 in our laboratory. The GCN (1.4×10^{11} ml^{-1}) for the resuspended virus pellets was approximately seven-fold higher than those calculated for $TCID_{50}$ (2.0×10^{10} ml^{-1}). A higher GCN ml^{-1} is to be expected since this will include RNA from virus without considering their infectivity. A real-time PCR assay will over-estimate the number of particles since viral RNA-target is also detected from free viral RNA from damaged cells and replication intermediates. In contrast, a $TCID_{50}$ assay will under-estimate the amount of virus particles since defective virus (non-infectious) are not evaluated. We therefore assessed that the number of viral particles in the pellet suspension was in between these two estimates (2.0×10^{10}–1.4×10^{11}).

3.2. Assay tested on plasma from fish experimentally challenged with SAV

To assess the xMAP assay for anti-SAV Ig detection under controlled conditions, we tested Atlantic salmon plasma from an experimental study with SAV2 and SAV3 [35,36]. Plasma was collected from co-habitants of SAV-injected shedders. At 6 wpc, data showed an increased Ig binding (detected as MFI) to beads coated with SAV whole virus (SAV1:1), compared to at 1 wpc (Fig. 1a). The beads coated with 1:10 dilutions of virus (not shown) gave similar results, although slightly lower antibody binding for all time points, compared to the 1:1 dilution. Beads coated with Triton-X-treated, disrupted SAV particles (SAV-TX) showed a significantly higher Ig binding at 2, 3 and 6 wpc compared to 1 wpc. Beads coated with recombinant SAV-E2 protein showed an increased binding to antibodies in plasma samples at 6 wpc, and a wider variation between samples at 1, 2 and 3 wpc compared to the other beads. Plasma from both SAV2- and SAV3-infected fish bound to all bead types in a similar manner. Control beads coated with uninfected CHSE cell lysates bound consistently low levels of antibodies in all tested plasma, with MFI values below 50 (Fig. 1b).

A few samples from SAV-injected shedder fish from the same challenge trial were also tested with the whole virus coated beads and the CHSE control beads. Again, the SAV-TX beads showed a higher Ig binding compared with SAV1:1 and SAV1:10, rising to an MFI of around 8000 for some fish, whereas binding to the CHSE control beads was consistently low at all time points (Fig. 2). Since both the SAV1:1 and the SAV1:10 beads were inferior to the SAV-TX beads, only the latter was used for SAV Ig detection in further analyses.

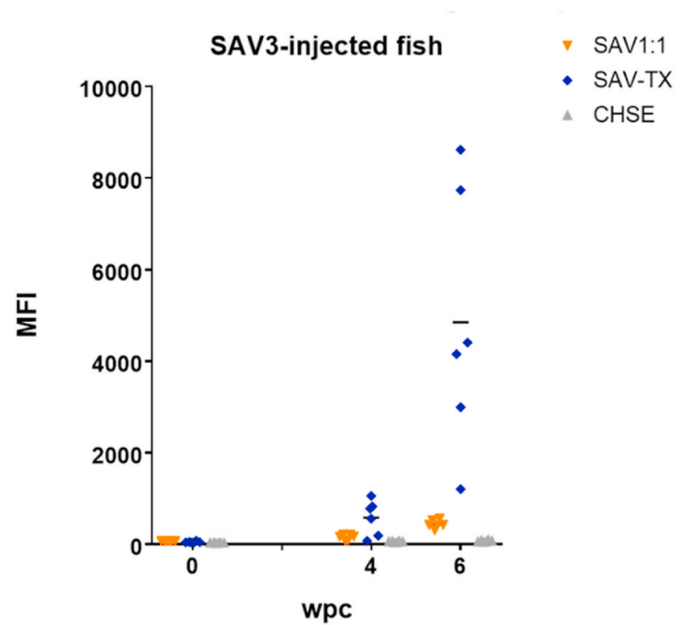

Fig. 2. SAV Ig detection in SAV-3 injected fish. Ig detected in plasma from Atlantic salmon injected experimentally (i.p) with SAV3 using an xMAP assay with beads coated with whole SAV3 particles (SAV 1:1) and disrupted SAV3 particles (SAV-TX) as antigen and CHSE control beads. MFI levels with mean.

3.3. Assay tested on plasma from fish experimentally challenged with PRV

To evaluate the specificity of the SAV antigens, the SAV-E2 and SAV-

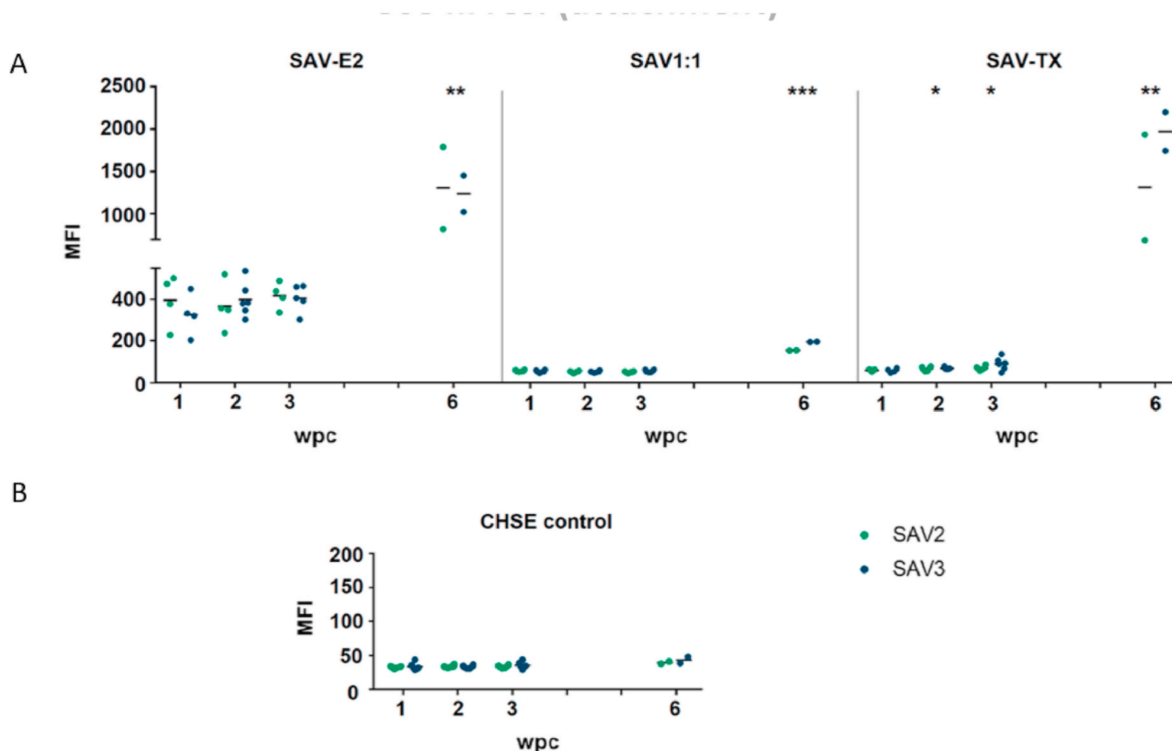

Fig. 1. Comparison of Ig binding to xMAP beads coated with SAV antigen.
A) Comparison between SAV3 E2 protein (SAV-E2), whole SAV3 particles diluted 1:1 (SAV 1:1) and Triton X-100 disrupted SAV3 particles (SAV-TX) coated on xMAP beads and used to test plasma from a SAV2/SAV3 cohabitant challenge for SAV-specific antibodies 1, 2, 3 and 6 weeks post challenge (wpc). B) Control beads coated with a lysate from the CHSE cells tested on the same plasma samples. MFI with mean values indicated by lines. Significant differences compared to 1 wpc are indicated by asterisks (Mann-Whitney test).

TX were tested on plasma from a PRV challenge trial together with other control antigens (Fig. 3). We have previously showed that a PRV infection induces production of non-specific, possibly polyreactive antibodies [27], and found high background binding to beads coated with the irrelevant antigens: lipid-modified ICP11 protein from shrimp white spot syndrome virus (ICP11), lipid-modified infectious salmon anemia virus fusion protein (FP-LM) and an unmodified infectious salmon anemia virus fusion protein (FP). In contrast to these proteins, the SAV-TX beads did not show any binding of non-specific Ig. SAV-E2 coated beads however, showed a similar background binding pattern as the irrelevant antigens.

3.4. Assay tested on plasma from fish undergoing a PD field outbreak

To assess the performance of these assays in aquaculture, samples from a field PD outbreak were analyzed using SAV-E2, SAV-TX and CHSE control beads as well as PRV1-μ1c beads used previously to detect PRV specific antibodies [26]. Plasma samples from 3 to 1 weeks before, and 2, 4, 6, 8, 10, 13 and 15 weeks after the defined start of the outbreak were analyzed. RT-qPCR showed viral RNA presence in fish from week 2 after the outbreak (Fig. 4a). The binding of plasma antibodies to SAV-TX beads started to increase from around two weeks after the PD outbreak, except for one fish (of the 10 tested) that was positive in the week before the outbreak (Fig. 4b). SAV-TX binding reached a maximum at around 8 weeks after the outbreak. At 13 and 15 weeks there were still many positive fish, while at these time points most of the samples were negative for SAV RNA by RT-qPCR (Fig. 4).

We detected antibodies binding to SAV-E2 beads from all time points. There was an increase in mean values from before the outbreak to 10 weeks after, similar to what was seen with SAV-TX, but the individual variations at each time point were very high (up to >10 000 MFI) even before the outbreak (Fig. 4b), and the increase was only statistically significant in week 10 and 13.

After the outbreak, all samples except four had neutralizing antibodies and/or virus in plasma (Fig. 5a). The presence of neutralizing antibodies could not be determined in viremic samples due to interference of the virus with the test principle. Neutralization titers and binding to SAV-TX followed roughly the same pattern, with antibody levels rising from around two weeks after the outbreak and staying elevated until the end of the sampling period, with some individuals deviating from this. Strong to moderate correlation between neutralization titers and MFI values of SAV-TX and SAV-E2 was found. A Spearman's correlation shows $\rho = 0{,}73$ ($p < 0{,}0001$) and $\rho = 0{,}43$ ($p = 0{,}0001$) for SAV-TX (Fig. 5b) and SAV-E2, respectively (Suppl. Fig. 2). There were, however, some individuals with high levels of neutralizing antibodies and very low levels of binding to SAV-TX and some negative for neutralizing antibodies with increased binding to SAV-TX. Weak correlations were found between neutralizing antibodies and binding to the other beads in the xMAP analysis (Suppl. Fig. 3).

Many of the field sample plasma from all time points contained antibodies binding to CHSE control beads. The levels were higher than in the challenge trial, but unlike the SAV-E2 and SAV-TX results there were no clear increase over this time period (Fig. 6a). A high degree of binding was also found to PRVμ1c and DNP-KLH coated beads (Fig. 6b and c).

By regression analysis, we found a correlation between Ig binding to CHSE control beads and SAV-E2 coated beads (Fig. 7). The binding to SAV-TX coated beads was not correlated to binding to the CHSE control beads (Fig. 7). Binding to DNP-KLH had a low correlation with SAV-E2 ($r^2 = 0{,}21$, $p > 0{,}0001$) and DNP-KLH beads had lower or no correlation with the other beads (Suppl. Fig. 4).

4. Discussion

In this study we found that a bead-based immunoassay with disrupted SAV particles was well suited to detect SAV-specific antibodies in plasma samples from both experimental infections and field outbreaks. Our results show that specific antibodies, covering both subtypes of IgM [41], could be detected from between 3 and 6 wpc in cohabitant fish and from around 4 wpc in SAV-injected fish. In the field outbreak of PD, a significant increase in SAV-specific antibodies was detected from two weeks after the defined start of the clinical outbreak. One fish also had increased antibody level one week before clinical PD was detected, probably reflecting that the virus had been present before infection was detected, and that a low prevalence of infected fish may occur for a period in the early stages of an outbreak. The level of antibodies in most samples remained elevated at the last sample point at 15 weeks after the outbreak, when only one sample had a low MFI. At this sampling point 70% of the samples were SAV negative by RT-qPCR.

Alphaviruses can be difficult to purify due to the fragility of the virion envelope [42]. A study comparing methods of density-gradient centrifugation of the alphavirus Semliki forest virus resulted in virus recovery of 6–40% [43]. Due to this great loss of virus particles we chose direct pelleting by ultracentrifugation as a method of concentration of SAV particles rather than purification through a gradient cushion. A higher concentration of virus particles was considered of greater importance than having a pure sample since the xMAP assay was run with a negative control of uninfected CHSE cells also pelleted by ultracentrifugation. We used a CHSE cell line (CHSE-ppG), that express the

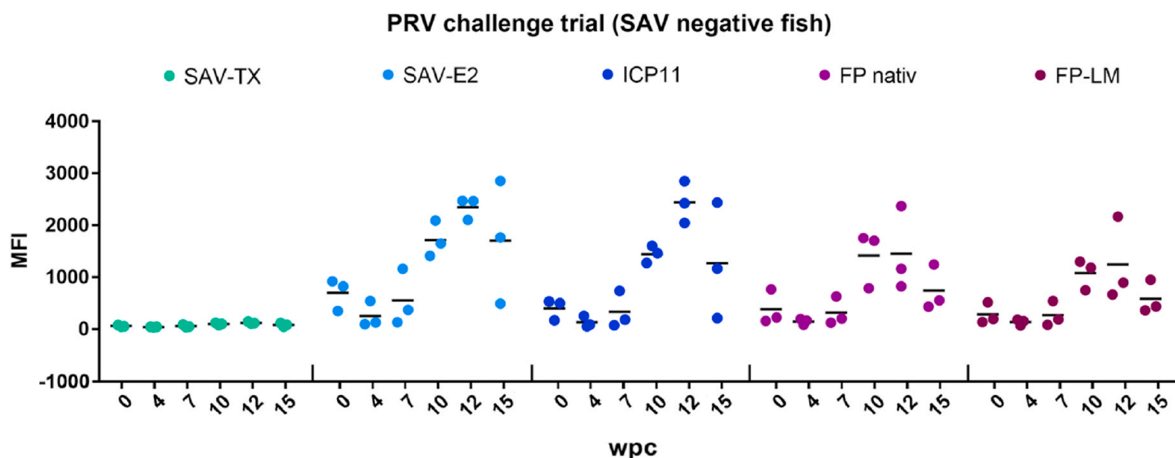

Fig. 3. Binding of non-specific Ig to beads coated with irrelevant antigens. Background Ig binding to beads coated with disrupted SAV particles (SAV-TX), recombinant E2 protein (SAV-E2) or antigens from other irrelevant viruses measured in plasma from an experimental PRV challenge trial. ICP11: Lipid-modified ICP11 protein from shrimp white spot syndrome virus, FP: Infectious salmon anemia virus fusion protein, FP-LM: Lipid-modified FP. MFI levels with mean.

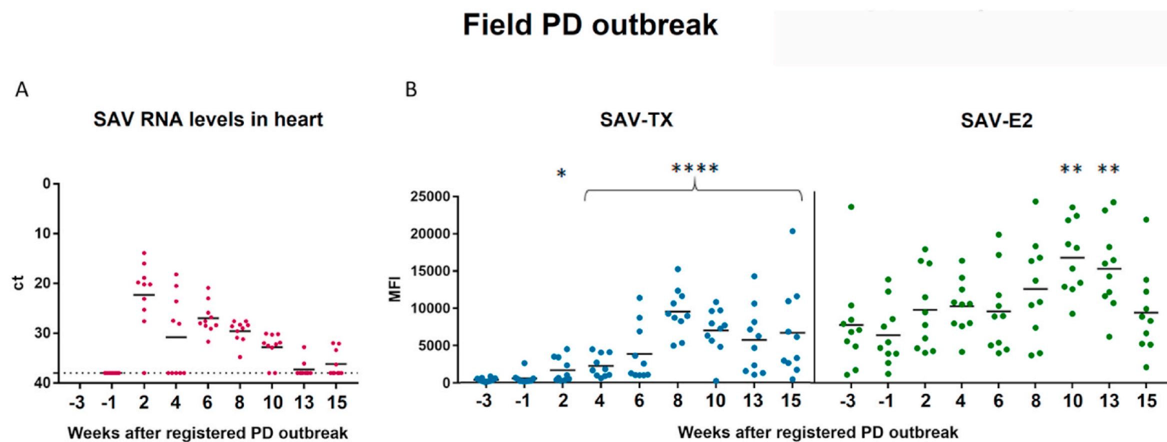

Fig. 4. Analysis of samples from a field outbreak of pancreas disease caused by SAV2. A) SAV RNA levels in hearts. Mean values indicated with lines. B) Levels of antibody binding to SAV-TX and SAV-E2 coated beads from plasma from the same outbreak. Mean values indicated with lines. Significant differences compared to three weeks before the outbreak are indicated by asterisks (Mann-Whitney test).

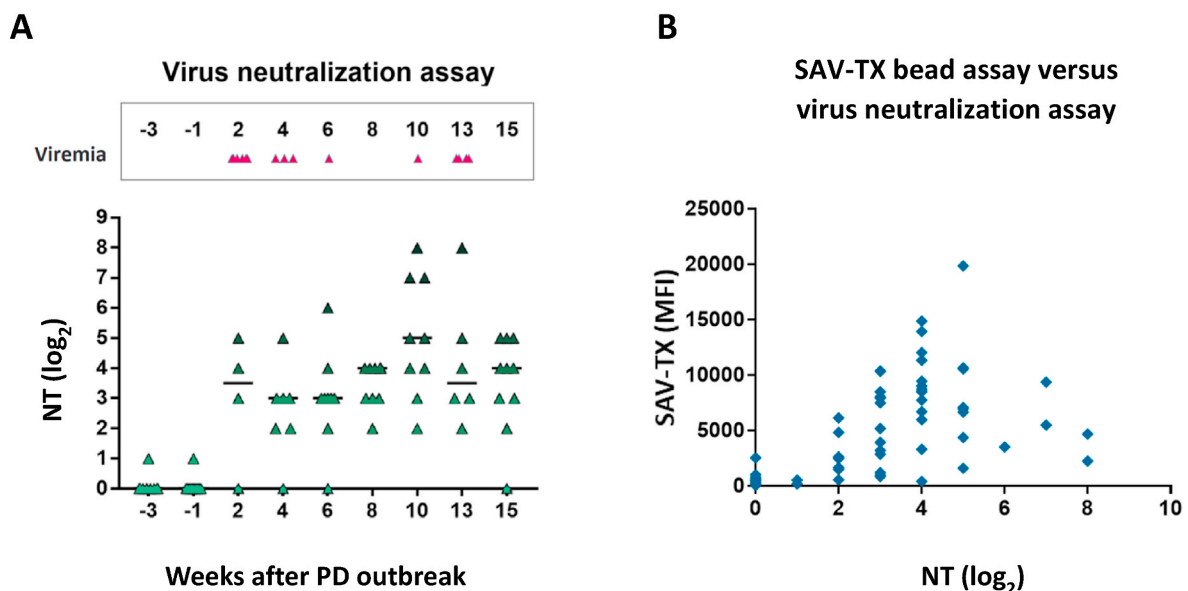

Fig. 5. Virus neutralization assay. A) Titer of neutralizing antibodies from a field PD outbreak. Median values indicated by lines. Neutralizing activity could not be determined in samples with viremia (in pink). B) Correlation between antibodies binding to SAV-TX-coated beads and neutralizing titers. NA; Neutralization Assay.

SAV-1 structural ORF, due to the missing ability of regular CHSE cells to produce large amount of virus when infected with a SAV3 isolate (H20/03). These cells are stably transfected with a plasmid that express the SAV-1 structural polyprotein with an N-terminal mEFGP tag. It is assumed that no viral-like particles are produced by these cells when uninfected, most likely because the mEGFP tag will interfere with the capsid construction, given that it is approximately the same size as the capsid protein (30 kDa). Since the capsid protein is immediately cleaved from the structural polyprotein after translation in the cytoplasm, the folding and structural conformation of E2 and E1 should not be affected, as this takes place in the endoplasmatic reticulum [12]. The limiting factors for SAV production in CHSE cells are unknown, but the increased access to E1 or E2 could be a positive factor in the production of virus particles, and an explanation why the CHSE-ppG produce more virus. The CHSE-ppG cells express SAV-1 structural polyprotein while the infectious virus added was a SAV3 subtype and it is not known if the virus particles produced in CHSE-ppG were SAV1/3 hybrids. However, due to the cross-neutralization between the SAV1-5 subtypes this would probably not influence the binding of specific antibodies against SAV3 or SAV2 [6]. The CHSE-ppG cells were therefore preferred for virus propagation to ensure sufficient amount of antigen for the xMAP assay.

The non-ionic detergent Triton-X was used to disrupt the virus particles. This method has been used previously to disrupt virus particles for bead-based immunoassays [44,45]. Triton-X solubilizes lipid-free membrane proteins without disruption of the native structure and biological function. Triton-X has been found to isolate but not separate E1 and E2 from the alphavirus Western equine encephalitis virus. The capsid was not affected by the treatment [46]. It is therefore likely that mainly intact E1-E2 heterodimers as well as virus capsids were present on our SAV-TX beads. This likely results in more available antigen on the SAV-TX beads compared to beads with untreated whole virus, explaining the increase in binding to SAV-TX compared to untreated SAV. The capsid protein may also contribute to the specific antibody binding to SAV-TX. Since it is not possible to distinguish which proteins the antibodies bind to, this work should be repeated with beads coated with purified recombinant capsid protein. This will show the ability of the capsid protein to bind specific antibodies against SAV.

Furthermore, we tested recombinant SAV-E2 protein as detection antigen and found binding of antibodies from experimentally SAV infected fish, albeit with a lower signal-to noise ratio. However, it also

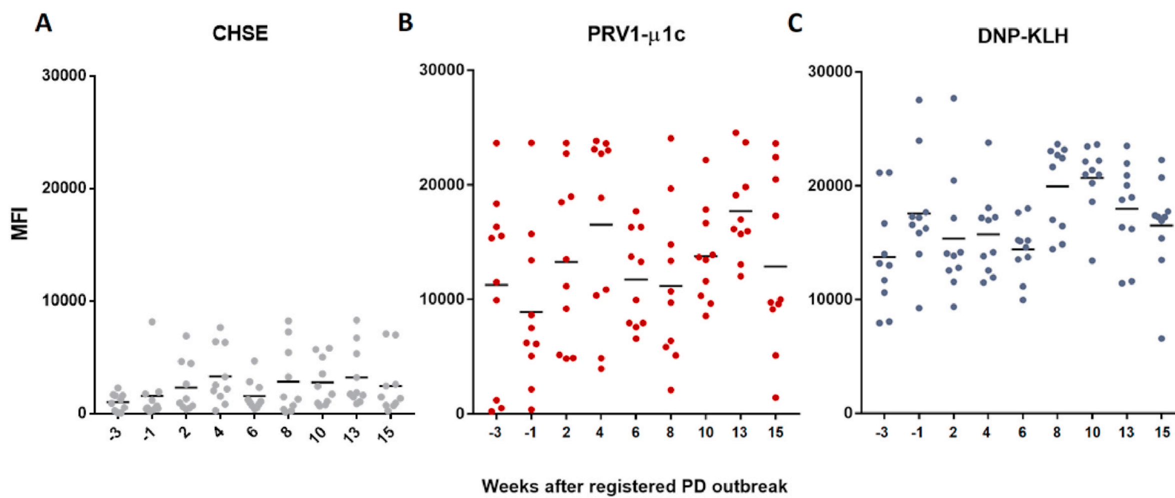

Fig. 6. Binding of plasma from PD outbreak to non-SAV antigens. A) CHSE control beads, B) PRV1-µ1c beads and C) DNP-KLH beads incubated with plasma from fish in the PD outbreak. MFI with mean.

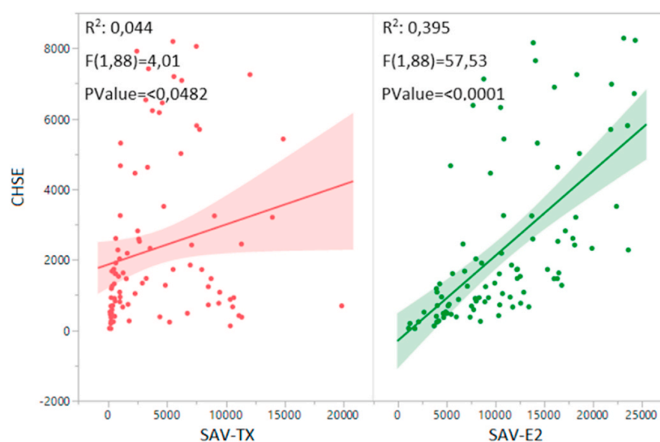

Fig. 7. Correlation between Ig binding to CHSE control and SAV-TX and SAV-E2 coated beads.

came out positive in PRV-infected, SAV-negative fish. The assay also gave a high background binding in pre-clinical samples from the PD field outbreak. High levels of non-specific, mostly polyreactive antibodies, are recognized as an important part of the humoral immune response in fish [40,47]. This antibody category may be induced after vaccination [39] or by infections [48], including PRV in Atlantic salmon [27]. Fish from the PD outbreak had been vaccinated with an inactivated vaccine against five bacterial diseases and infectious pancreas necrosis virus. Furthermore, these fish were PRV-infected, as they tested positive for antibodies against PRV protein µ1c at all time points, and the farm from which these fish originated had a diagnosis of HSMI six months prior to the first positive SAV sampling. Plasma binding to SAV-E2 correlated with binding to CHSE cells, indicating non-specific binding properties. However, binding to SAV-E2 did not correlate well with binding to DNP-KLH, often used to measure non-specific antibodies [39,40]. Also, binding to SAV-E2 increased during the PD outbreak, not seen with DNP-KLH or PRV-µ1c beads, indicating that at least a proportion of the antibodies binding to SAV-E2 represented SAV-specific binding. A much clearer picture emerged with SAV-TX coated beads, which did not result in any detectable cross-reactivity when testing plasma from the experimentally PRV-challenged fish, or in the field material prior to PD outbreak, despite a high presence of non-specific antibodies in both circumstances. This indicates that the SAV-TX antigens primarily bind highly specific antibodies against SAV and would be the antigen of choice for this immunoassay.

The non-specific binding to the recombinant E2 protein illustrates a pitfall when selecting purified antigens for use in immunoassays for fish. The currently used antigen was expressed in a bacterial system, which have the disadvantage of growth at 37 °C, the lack of glycosylation by the bacterial host and aberrant post-translational modification. The E2 protein has been shown to require co-expression with E1 and lower temperatures for correct folding and for translocation and presentation on the cell surface [49]. Furthermore, there may be arguments to avoid the usage of E2 protein alone. In alphaviruses infecting mammals, antibodies against E2 are more often neutralizing than antibodies binding to E1 [50,51]. However, when studying both IgM and IgG produced after infection with the alphavirus *Chikungunya virus* in humans, Chua and co-workers found that while neutralizing IgG bound to epitopes on E2, neutralizing IgM bound to specific conformation-dependent epitopes on the E1-E2 heterodimers [52]. This could also be the case in salmon as IgM is the dominating circulating antibody. Experimental SAV vaccination showed that whole structural polyprotein immunization may be required to obtain neutralizing antibodies and protection [25]. In contrast, a DNA vaccine encoding only the E2 protein provided no antibodies against E2, no neutralizing antibodies, no reduction in pathology and no reduction of virus level in serum. In another vaccine challenge comparing DNA vaccines and subunit vaccines with E1 or E2 and an inactivated vaccine, levels of antibodies against E2 did not correlate with level of neutralizing antibodies or reduction of mortality [24]. Taken together, an intact E1-E2 complex in its native conformation may be critical for the detection of specific SAV antibodies that correlate with protection.

SAV neutralization assays can be considered a gold standard for the inhibitory effects of SAV-specific antibodies on viral infection [15], and a good control for the immunoassay. Despite some discordant individuals, we found a strong correlation between binding of antibodies to SAV-TX beads and neutralization titers. Whereas neutralizing antibodies are expected to target specific epitopes on the SAV E-proteins, the SAV-TX beads may also detect antibodies binding to the capsid and any part of the E-proteins. In contrast, there was only a moderate correlation between SAV-E2-binding and neutralizing antibodies. The binding to the SAV-E2 beads are likely a mixture of SAV-specific antibodies against linear epitopes and non-specific, possibly polyreactive antibodies. While neutralizing antibodies can work in several different ways to block infectivity, non-neutralizing antibodies can control infection by antibody-dependent cellular toxicity, complement-mediated lysis with the formation of a membrane attack complex, and Fc receptor-mediated phagocytosis. The relevance of the neutralizing and the specific

antibodies in relation to protection against PD requires further research.

To ensure management of PD in Norwegian aquaculture, the Norwegian coastline is divided into different zones by fish health authorities, with the aim to control SAV infection in farmed fish. Serological methods can have advantages for surveillance in addition to the virus screening that is currently performed [14]. Immunoassays may detect infection after the peak phase and previous virus encounter longer than the virus can be detected in the fish, as shown in this work, and in previous studies [17,53]. The xMAP method is time saving compared to virus neutralization assays and RT-qPCR. The small amounts of sample material needed for xMAP analyses would also make it suitable for non-lethal testing and thereby testing of more fish in a surveillance program, especially to document freedom from SAV outside of endemic zones. Non-lethal sampling for antibodies could be done when the fish are handled for lice counting or other procedures. Because of large individual variations in binding and partly unresolved specificities, serological testing of salmon for SAV infection should primarily be used for groups of fish and not individuals. Plasma from several fish could also be pooled. When using pooled samples, more fish can be tested, and the distribution of SAV can be better monitored, even when the prevalence is low. More testing of the xMAP assay, including on subclinical SAV infections, later than 15 weeks after the start of a PD outbreak, with pooled samples and on SAV-vaccinated fish, should be done to confirm that this method is at least as sensitive and specific as neutralization assays.

Special caution must be taken with serological assays in fish due to the relatively high presence of non-specific antibodies. Factors leading to high non-specific antibody titers may be a history of vaccination and concurring infections; in Norwegian field samples PRV must be expected [54,55]. The results presented here demonstrate that a high background of non-specific antibodies must be considered when selecting antigens and interpreting results. Finding good antigens that bind specifically with limited binding to non-specific antibodies is a significant challenge for serological tests in Atlantic salmon. The present study indicates that Triton-X-treated whole SAV particles may indeed fulfill this goal for SAV detection. Further studies to verify the sensitivity and specificity of this test system under different practical field situations is warranted before employing the method into routine use.

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CRediT authorship contribution statement

Lena Hammerlund Teige: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Visualization, Writing - original draft, Writing - review & editing. **Ida Aksnes:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Visualization, Writing - original draft, Writing - review & editing. **Magnus Vikan Røsæg:** Resources. **Ingvill Jensen:** Resources. **Jorunn Jørgensen:** Resources. **Hilde Sindre:** Investigation. **Catherine Collins:** Resources. **Bertrand Collet:** Resources. **Espen Rimstad:** Supervision, Writing - review & editing. **Maria K. Dahle:** Supervision, Writing - review & editing. **Preben Boysen:** Supervision, Visualization, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.07.055.

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