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Rainbow trout surviving infections of viral haemorrhagic septicemia virus (VHSV) show lasting antibodies to recombinant G protein fragments

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ABSTRACT

Rainbow trout antibodies (Abs) binding to recombinant fragments (frgs) derived from the protein G of the viral haemorrhagic septicemia virus (VHSV)-07.71 strain, could be detected by ELISA (frg-ELISA) in sera from trout surviving laboratory-controlled infections. Abs were detected not only by using sera from trout infected with the homologous VHSV isolate but also with the VHSV-DK-201433 heterologous isolate, which had 13 amino acid changes. Sera from healthy trout and/or from trout surviving infectious haematopoietic necrosis virus (IHNV) infection, were used to calculate cut-off absorbances to differentiate negative from positive sera. Specific anti-VHSV Abs could then be detected by using any of the following frgs: frg11 (56–110), frg15 (65–250), frg16 (252–450) or G21-465. While high correlations were found among the ELISA values obtained with the different frgs, no correlations between any frg-ELISA and complement-dependent 50% plaque neutralization test (PNT) titres could be demonstrated. Between 4 and 10 weeks after VHSV infection, more trout sera were detected as positives by using heterologous frg-ELISA rather than homologous PNT. Furthermore, the percentage of positive sera detected by frg11-ELISA increased with time after infection to reach 100%, while those detected by complement-dependent PNT decreased to 29.4%, thus confirming that the lack of neutralizing Abs does not mean the lack of any anti-VHSV Abs in survivor trout sera. Preliminary results with sera from field samples suggest that further refinements of the frg-ELISA could allow detection of anti-VHSV trout Abs in natural outbreaks caused by different heterologous VHSV isolates. The homologous frg-ELISA method could be useful to follow G immunization attempts during vaccine development and/or to best understand the fish Ab response during VHSV infections. The viral frgs approach might also be used with other fish species and/or viruses.

1. Introduction

The *in vitro* complement-dependent neutralization tests (50% plaque neutralization test, PNT) are usually employed to estimate protein G-specific antibodies (Abs) to viral haemorrhagic septicemia virus (VHSV) [3,11,12,24]. In addition to complement-dependent PNT being highly specific and sensitive, detection of neutralizing Abs generally correlates well with protection to VHSV challenge [17,19,20]. However, PNTs are also time-consuming, labour-intensive and require laboratories with cell culture facilities [16,25]. Most important, because the VHSV isolate used for the complement-dependent PNT needs to be homologous or closely related to the isolate causing the infection, the assay is being prone to false negatives when applied to natural infections, since it is not always possible to match both isolates [1,9,10].

Binding to VHSV-captured enzyme linked immunosorbent assays (ELISA) has been alternatively used to estimate anti-VHSV Abs whether those be neutralizing or not [25]. Other attempts to detect trout anti-VHSV protein G (G) Abs have used purified VHSV or yeast recombinant protein G4 (amino acid, 9–443) to coat solid phases for indirect ELISAs [6]. However, some of these assays suffered from either high backgrounds and/or low sensitivities and the correlation of their results with protection has not been supported by any data [17,19,20].

To search for possible G short fragments (frgs) as alternative solid phases for indirect ELISAs, a 15-mer overlapping pepscan peptide library of the G protein of VHSV-07.71 was screened for trout Ab reactivity. However, it was not possible to map neutralizing

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monoclonal Abs by this method and very few peptides specifically reacted with homologous anti-VHSV trout sera [8]. Because, those results could have been due to the small size of the peptides used, trout Ab reactivity was then searched in larger G frgs. Thus the recombinant frg11 (amino acid 56–110) implicated in VHSV fusion [7], was another alternative used to estimate anti-G binding Abs [8,28].

Here we have screened sera from trout surviving laboratory-controlled infections with homologous and heterologous VHSV isolates as a first step to explore the possibility of using recombinant G frgs to estimate the presence of anti-VHSV Abs in sera from trout surviving natural infections independently of their neutralizing or non-neutralizing activities. Thus, several recombinant frgs were derived from the VHSV-07.71 french isolate, while the sera were obtained from trout infected with either homologous (VHSV-07.71) or heterologous isolates (VHSV-DK-201433 from Denmark, differing in the protein G sequence by 13 amino acids from VHSV-07.71). In contrast to the Abs detected by complement-dependent PNT, Abs detected by frg-ELISA were found in more sera from trout infected not only with the homologous but also with the heterologous isolate under laboratory-controlled conditions. The results showed that more positive sera could be detected by frg-ELISA than by complement-dependent PNT at any time after infection. Furthermore, while the number of sera with positive ELISA increased, those with positive PNT decreased with time after infection, thus showing that the lack of neutralizing Abs does not mean there are no other types of anti-VHSV Abs present in survivor trout sera. Preliminary results obtained with a limited amount of field samples suggest that further refinements could allow detection of anti-VHSV trout Abs in natural outbreaks caused by different heterologous VHSV isolates. This method will be useful to follow immunization attempts during vaccine development and/or the kinetic of fish Ab response following homologous VHSV infections. The antigenic viral frgs approach might also be used with other fish species and/or viruses.

2. Materials and methods

2.1. Construction of recombinant fragments (frgs) of the glycoprotein G of viral haemorrhagic septicemia virus (VHSV)

The constructs, expression and purification of frgs of the protein G of VHSV were performed as described [28]. Briefly, the G protein sequence (Gene Bank accession number X59148) of the VHSV-07.71 strain, isolated in France from rainbow trout (*Oncorhynchus mykiss*) [14,30] was used to design the frgs (G21-465, frg11, frg15 and frg16). Their sequences were then chemically synthesized and cloned into pRSET expression vectors that add poly histidine tails. The G21-465 was expressed in *Trichoplusia ni* (cabbage looper) insect larvae [22,27] while the other fragments frg11 (56–110), frg15 (65–250) and frg16 (252–450) were expressed in *Escherichia coli*. Purification of the recombinant frgs was made by Ni^{2+} affinity chromatography followed by Sephadex chromatography as described [28].

2.2. Confirmation of recognition of G frgs by western blotting with trout sera

Confirmation of fragment recognition by trout Abs was performed by western-blot on SDS-15% polyacrylamide gels in buffer containing 2-β mercaptoethanol. The proteins in the gel were transferred to nitro-cellulose membranes (BioRad, Richmond,Vi) and blocked with dilution buffer (0.5% bovine serum albumin, 0.1% Tween-20, 0.01% merthiolate, 0.005% phenol red in phosphate buffered saline pH 6.7). Three mm wide strips were incubated with the corresponding trout Ab and with the peroxidase-conjugated rabbit-anti-mouse (RAM-PO) Ab and developed with diaminobenzidine (DAB) staining.

2.3. Rainbow trout sera used for the screening

Rainbow trout *O. mykiss* (Walbaum) of ~50 g obtained from a certified VHS- and IHN-free trout farm were infected in Denmark under laboratory-controlled conditions (12 ± 2 °C with constant aeration) by immersion for 2 h with 2×10^5 TCID50 per ml of water of VHSV-201433-40 isolated from rainbow trout originating from an infected farm in Denmark [9,10]. A total of 77 sera samples were collected from survivor trout after 4 ($n = 23$), 6 ($n = 21$), 8 ($n = 16$) and 10 ($n = 17$) weeks. Five sera sampled from rainbow trout 12 weeks after infection with VHSV-07.71 [14] by immersion and 14 sera sampled from rainbow trout 4 weeks after intramuscular injection, were kindly provided by Dr. Jeannette Castric (AFSSA, now ANSES, France). In order to estimate cut-off values, non-infected trout sera were collected from 15 healthy trout (500 g of body weight) maintained in a closed-circuit farm with no previous history of VHSV infections for more than 30 years (Escuela de Montes, Madrid, Spain). The non-infected sera cut-off values were used to study both the sera from VHSV-infected rainbow trout and 45 sera collected from rainbow trout surviving infection with IHNV strain 039-82 in USA with neutralization titres >200 kindly provided by Dr. Scott LaPatra (Clear Spring Foods, Inc, Idaho USA). Rainbow trout sera ($n = 20$) were collected from two farms in different Denmark locations with high (~80%) and medium (~50%) mortalities produced by VHSV outbreaks in 2006 (farm A 206277) and in 2008 (farm B 2008-50-362), respectively.

2.4. frg-ELISA assays of rainbow trout sera

Polystyrene plates of Maxisorb 96-wells (Nunc Inmunoplates) were used as ELISA plates. The wells were coated with 5 μg per well of purified recombinant frg11 (56–110), frg15 (65–250), frg16 (252–450) or G21-465 in 100 μl 0.1 M carbonate buffer pH 9.6 and incubated overnight at 4 °C. To reduce the background, the coated wells were then blocked with 100 μl per well of dilution buffer (0.5% bovine serum albumin, 0.1% Tween-20, 0.01% merthiolate, 0.005% phenol red in phosphate buffered saline pH 6.7) containing 0.025% of skimmed milk, and washed with 0.5% Tween-20 in water before performing the ELISA. The wells were incubated during 60 min at room temperature with 100 μl/well of 200-fold diluted trout sera in dilution buffer. After washing, they were incubated during 30 min with anti-trout immunoglobulin MAb 1G7 mouse ascites diluted 1000-fold [31]. After washing, 5000-fold diluted horseradish peroxidase-conjugated rabbit-anti-mouse antibody RAM-PO (Sigma Chem.Co, St.Louis, Mo) was added. After one more wash, a final incubation of 30 min with 0.5 M NaCl 0.01% SDS was required when using frg15, frg16 and G21-465 to reduce their backgrounds. The colour reaction was developed by adding 1 mg/ml o-phenylenediamine in citrate buffer containing 3 mM H_2O_2. Optical density was measured by using dual wave length at 492–620 nm in a Labsystems ELISA reader. The optical density at 620 nm was used to correct for individual differences between wells as recommended by the manufacturer. Absorbances obtained in fragment coated wells that received no trout sera were subtracted from all the values (background absorbances varied from 0.05–0.15, depending on the fragment). To estimate frg-ELISA titres in large numbers of trout sera, the expression of the results as the absorbance obtained at a unique 200-fold dilution (fixed dilution) rather than the first 2-fold dilution that reached background absorbances (limiting dilution titre) was chosen (Fig. 1). To correct for possible unspecific binding of rainbow trout sera obtained from field VHSV outbreaks, frg11-ELISA was carried out in parallel with

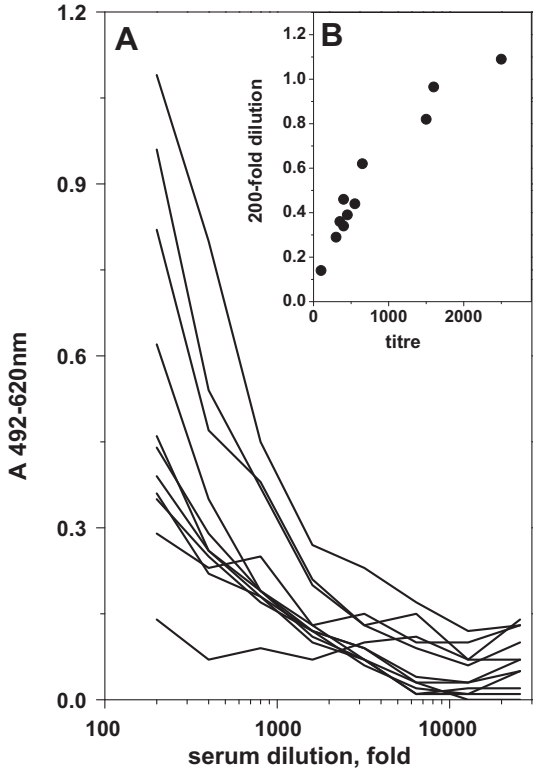

Fig. 1. Variation of frg11-ELISA absorbance values with dilution of VHSV-infected trout sera (A) and comparison of titres estimated by limiting and fixed dilution methods (B). Trout sera representing a whole range of different titres were selected for ELISA on plates coated with frg11 (frg11-ELISA) derived from the protein G of VHSV-07.71. Trout sera were 2-fold serially diluted from 200 to 25,600-fold and ELISA performed as indicated in methods. A, absorbances at 492–620 nm of serial dilutions of 11 trout sera. B, comparison of ELISA titres defined either as the dilution required to obtain the cut-off of 0.24 units of absorbance (titre) or the absorbance value at a 200-fold dilution.

plates coated with 5 μg per well of either frg11 or bovine serum albumin (BSA). The absorbance values obtained in plates coated with BSA were substracted to the absorbance values obtained with plates coated with frg11.

2.5. VHSV complement-dependent 50% plaque neutralization test (PNT)

The complement-dependent 50% PNT was performed in 96-well plates as previously described [9]. Briefly, 50 μl of serial 2-fold dilutions of previously heat inactivated trout sera in dilution medium (Eagles MEM with Tris buffer and 5% fetal bovine serum), were mixed in round bottom 96-wells with 50 μl of fresh 30-fold diluted trout sera as a source of complement and incubated for 30 min at 15 °C on a rocker platform. Then, 100 μl of 8×10^3 pfu of the corresponding homologous VHSV isolate (either 07.71 or 201433) per ml were added to each well (the homologous isolate was chosen to maximize *in vitro* neutralization) and the plate incubated overnight at 15 °C on a rocker platform. Each serum-complement-virus mixture was then adsorbed to 2 replicate wells (10 μl per well) with a monolayer of EPC cells for 1 h at 15 °C. The cell cultures were then overloaded with cell culture medium containing 1% methyl cellulose and incubated for 5 days at 15 °C. Finally, the cells were fixed with 10% phosphate buffered formaldehyde, washed and stained with 0.5% crystal violet. After washing, the plates were air-dried and the lysis plaques counted. The complement-dependent 50% PNT titre was calculated as the reciprocal value of the highest trout serum dilution causing a 50% reduction of the average number of plaques in control cultures inoculated with non-infected trout serum. Because some trout sera at low dilutions show cellular toxicity, a cut-off value of 80 was chosen to discriminate negative from positive neutralization titres.

3. Results

Preliminary experiments showed that rainbow trout antibodies (Abs) against the G protein of the french VHSV strain 07.71 (VHSV-07.71) could be estimated in sera from trout repeatedly injected intraperitoneally with both purified VHSV and G protein (hyper-immunized trout) by using the homologous recombinant fragments (frgs) as solid phases for ELISA (frg-ELISA) (data not shown). Experiments using a few sera from trout surviving laboratory-controlled infections with the DK-201433 Danish VHSV heterologous isolate (differing with 13 amino acid mutations from the VHSV-07.71 isolate) suggested that the corresponding frg-ELISA could also be used to estimate the Ab levels in those sera. In both of the above mentioned cases, Western blotting confirmed that the absorbances obtained by frg-ELISA corresponded to true reactivity with the frgs. Thus, pooled sera from trout infected either with VHSV-07.71 having >10,000 ($n = 4$) and <80 ($n = 3$) PNT titres or with VHSV-201433 having >10,000 ($n = 4$) and <80 ($n = 4$) PNT titres, reacted equally well with frg11, frg15, frg16 or G21-465 by western blotting. In contrast, pooled non-infected trout sera ($n = 15$) did not recognized any of the frgs (data not shown).

To semiquantitatively estimate frg-ELISA titres in large numbers of individual trout sera, the results obtained by using the limiting dilution (the first dilution to reach background absorbances) and fixed dilution (the absorbance at 200-fold dilution) methods were compared. Because the comparison of 11 trout sera selected within a wide range of frg11-ELISA titres (Fig. 1A), showed a linear correlation between the results obtained with both methods (Fig. 1B), the much simpler estimation of absorbances at the 200-fold dilution method was chosen to continue the experimentation.

To select the most appropriated recombinant frg of the protein G of VHSV to assess the presence of anti-VHSV Abs, trout sera were analysed by frg-ELISA by using solid phases coated with frg11, frg15, frg16 or G21-465 from VHSV-07.71. First, to distinguish negative from positive sera (cut-off values), sera from non-infected and IHNV-infected trout were compared with sera from VHSV-201433 infected rainbow trout. Sera from non-infected trout ($n = 15$) showed a calculated cut-off absorbance value of 0.24. On the other hand, the distribution of absorbance values of sera from IHNV-infected trout ($n = 45$) contained 6 sera (13.3%) with absorbance values >0.5. Whether those were due to crossreactivity between the G proteins of IHNV and VHSV or to Abs to VHSV was not investigated (there were considered as outliers). The inclusion of the remaining 39 sera from IHNV-infected trout to the 15 sera from non-infected trout to re-calculate cut-offs, resulted in a mean absorbance of 0.06 which corresponded also to a calculated cut-off value of 0.24 (mean + 2 standard deviations) (not shown). In contrast, Fig. 2 shows the distribution to higher absorbance values of most of the sera from VHSV-201433 infected trout (0.46 ± 0.24, $n = 77$). Similar profiles but differing in their cut-off values were obtained when using frg15, frg16 or G21-465 as solid phases (data not shown).

Once the cut-off values for each frg were estimated, individual sera from trout harvested at different times after VHSV-201433 infection were assayed. The resulting absorbance values of trout sera infected with VHSV-201433 estimated with frg15, frg16 or G21-465 showed linear correlation coefficients of 0.91, 0.82 and 0.85, respectively when compared with those obtained with frg11 (Fig. 3). A correlation coefficient of 0.84 was obtained when comparing 2 different experiments with frg11 (not shown). Similar

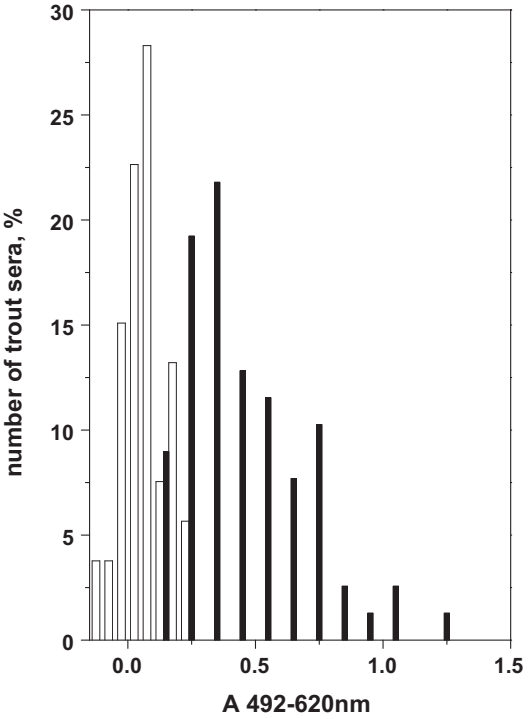

Fig. 2. Distribution of frg11-ELISA values from non-infected + IHNV-infected and VHSV-infected trout sera. Sera from non-infected ($n = 15$) and IHNV-infected rainbow trout ($n = 39$) were used as negative specific controls for anti-G VHSV Abs. A total of 77 rainbow trout sera were obtained from VHSV-201433 infected rainbow trout and assayed. All the sera were 200-fold diluted and frg11-ELISA performed as indicated in methods. Absorbance values were classified in 0.1 absorbance classes and the resulting grouping represented. Open bars, sera from non-infected and IHNV-infected rainbow trout. Black bars, sera from VHSV-201433-infected rainbow trout.

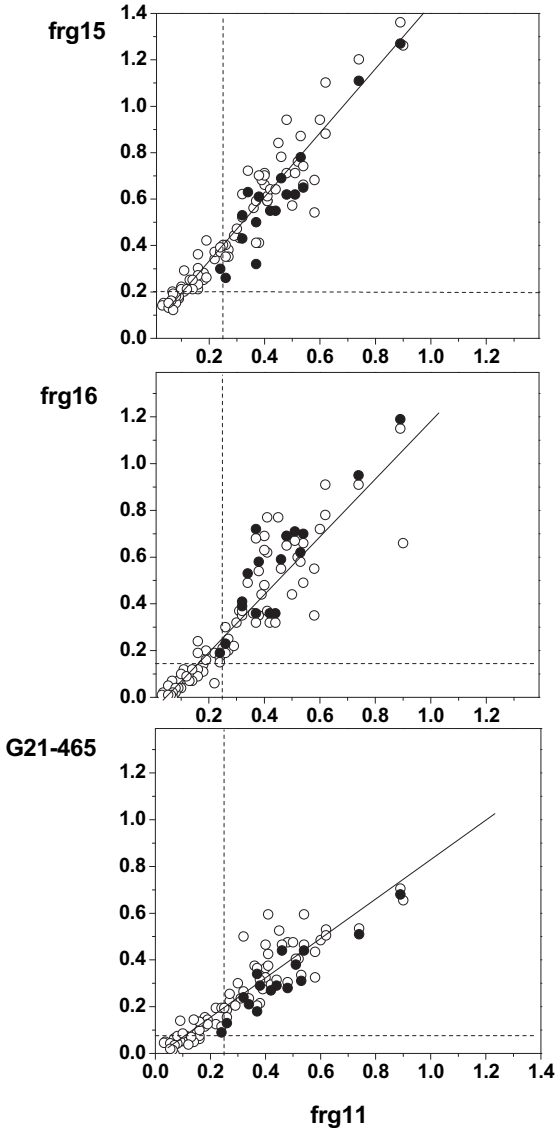

Fig. 3. Comparison between frg-ELISAs absorbance values obtained with different G fragments by using VHSV-201433 survivor sera. Sera from rainbow trout surviving VHSV-201433 infection were diluted 200-fold and assayed by ELISA by using solid phases coated with purified frgs derived from the protein G of VHSV-07.71. The values obtained with frg15, frg16 and G21-465 were compared with the values obtained with frg11. Two experiments were performed with similar results, one of them is shown in the figure. ———, cut-off values were calculated by the following formula, mean absorbance of sera from IHNV-infected ($n = 39$) and non-infected ($n = 15$) rainbow trout + 2 × standard deviations. Straight line, linear fitting of the absorbance values obtained for the VHSV-201433 sera. ○, sera from rainbow trout harvested between 4 and 10 weeks after VHSV-201433 infection ($n = 77$). ●, sera from rainbow trout harvested 10 weeks after VHSV-201433 infection ($n = 17$).

results were obtained when using sera from trout infected with VHSV-07.71 (not shown). In contrast, the frg-ELISA absorbances of the sera did not correlate with their harvest time after VHSV infection. Thus, as one example, the distribution of values obtained from sera harvested 10 weeks after VHSV infection showed values ranging from the lowest to the largest frg-ELISA absorbances (Fig. 3, black circles). Similar results were obtained with the sera obtained at other times after infection (Fig. 3, data not differentially labelled). Because frg11 was the smallest G frg located at a region with the lowest amino acid variability [2,4,29] and showed the lowest background values, it was chosen to carry out the rest of the analysis with this frg.

Fig. 4 shows the comparison between the titres obtained from frg11-ELISA and complement-dependent PNT. There is no correlation between frg11-ELISA and PNT values for the sera from trout infected either with VHSV-07.71 (Fig. 3, open symbols), nor with sera from VHSV-201433 infected trout (Fig. 3, black symbols). While a total of 90.1% of the sera analysed from VHSV-infected trout were >0.24 by frg11-ELISA, only 45% had PNT titres >80.

The percentages of sera from VHSV-201433 infected trout at different times after infection were then classified in positive/negative classes by their complement-dependent PNT or frg11-ELISA ($n = 77$) titres. From 4 to 10 weeks after VHSV infection, the percentage of the positive sera estimated by frg11-ELISA increased from 86.9 to 100% (Fig. 5 black circles) while by PNT decreased from 65.2 to 29.4% (Fig. 5 black squares). Furthermore, 10 weeks after infection 100% of the sera which were positive had absorbance values >0.4. A total of 38.9% of the sera that were negative by PNT were positive by ELISA, while a total of 3.3% of the sera that were negative by ELISA were positive by PNT. Only 7.8% of the VHSV-201433 infected sera were negative for both ELISA and PNT and all those sera were found at the earliest times after infection (4–6 weeks).

To preliminarily evaluate the potential use of frg11-ELISA to detect previous exposure to different VHSV isolates in different locations after natural infections, rainbow trout sera samples from 2 different farms were collected after their corresponding VHSV outbreaks caused either high or medium mortalities (~ 80% farm A and ~50% farm B, respectively) and assayed by PNT, VHSV-ELISA and frg11-ELISA. To increase the sensitivity of the PNT, the cut-off was established at a <40 dilution and several VHSV isolates were used for the test. To increase specificity of the frg11-ELISA, the

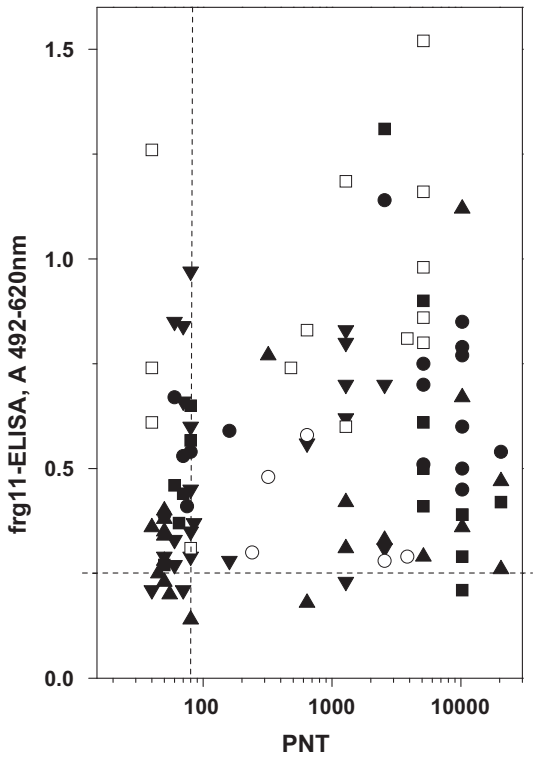

Fig. 4. Lack of correlation between frg11-ELISA and complement-dependent 50% PNT titres of VHSV-infected rainbow trout sera. Plates used for frg-ELISA were coated with VHSV-07.71 derived frg11. Rainbow trout were infected with VHSV-07.71 (open symbols) or VHSV-201433 (black symbols) and their sera harvested at different times (4–10 weeks) after infection. Sera were 200-fold diluted and ELISA performed as indicated in methods and their means represented ($n = 2$). Complement-dependent 50% PNT was performed as indicated in methods. To show all the PNT values <80 in the figure, they have been arbitrarily modified in ±10 increments. ▼, 4 weeks after infection with VHSV-201433. ▲, 6 weeks after infection with VHSV-201433. ■, 8 weeks after infection with VHSV-201433. ●, 10 weeks after infection with VHSV-201433. □, 4 weeks after intraperitoneal injection of VHSV-07.71. ○, 12 weeks after infection with VHSV-07.71. ———, cut-off values of 80 for PNT and 0.24 for frg11-ELISA.

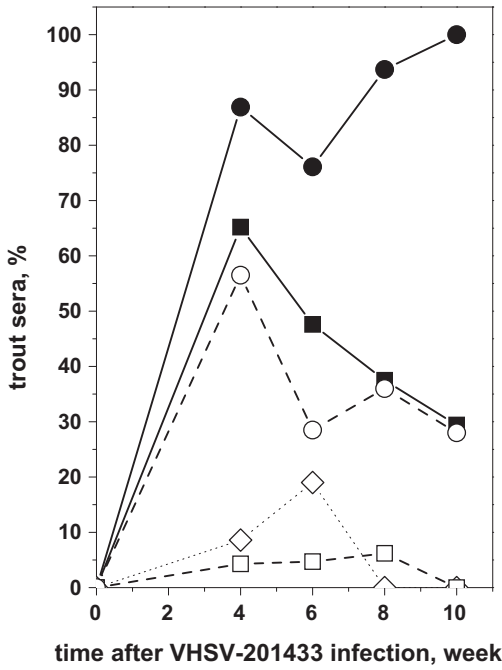

Fig. 5. Time course of the percentage of positive/negative sera from rainbow trout infected with VHSV-201433 by frg-ELISA and complement-dependent 50% PNT of data from Fig. 4. Rainbow trout were infected with VHSV-201433 and sera from survivors harvested 4 ($n = 23$), 6 ($n = 21$), 8 ($n = 16$) and 10 ($n = 17$) weeks after infection. A 0.24 absorbance value and a dilution of 80-fold were used as the cut-offs to differentiate and group negative from positive sera by frg11-ELISA and complement-dependent PNT, respectively. Percentages were calculated by the formula, 100 × number of sera according to the specified criteria (see symbol explanations)/total number of sera analysed at any given time point after infection. ●, percentage of sera that were frg11-ELISA positive. ■, percentage of sera that were PNT positive. ○, percentage of sera that were both PNT negative, frg11-ELISA positive. □, percentage of sera that were both PNT positive, frg11-ELISA negative. ◇, percentage of sera that were both PNT negative, frg11-ELISA negative.

absorbance values obtained by coating the plates with BSA were subtracted from the absorbance values obtained with plates coated with frg11. Results in farm A confirmed that best sensitivity for PNT was obtained when using the homologous isolate for the test (70% positives) while only 50 or 15% positives could be detected when using a related isolate or the F25 strain, respectively. Similar (65%) or lower (40%) percentages of positive sera were obtained when using VHSV or frg11-ELISA, respectively, for the sera from this farm (Table 1). In contrast, in farm B only 15% of the sera could be detected as positives by either homologous PNT or VHSV-ELISA while up to 80% of the sera resulted positive by frg11-ELISA.

4. Discussion

The highest percentage of positive sera by frg-ELISA from trout surviving a VHSV infection under laboratory-controlled conditions were found 10 weeks after infection (100%) in contrast to the positive sera by complement-dependent PNT (29.4%). Those results show that the absence of neutralizing Abs does not mean that other anti-VHSV binding Ab were absent in sera from surviving rainbow trout, therefore confirming earlier observations that showed non-neutralizing Abs tend to persist longer than neutralizing Abs in VHSV survivors [5,21,25]. However, since frg-ELISA is an *in vitro* method, it remains unknown whether or not the newly detected binding Abs are protective *in vivo*. Previous estimations of binding Abs by using VHSV-captured ELISA did not correlate with protection [17,19,20], however it might also be that the new binding Abs detected by frg-ELISA might recognize other additional epitopes not yet studied. On the other hand, because at least one *in vitro* non-neutralizing monoclonal Ab was capable of providing *in vivo* passive protection [18], it might also be possible that some of the binding Abs which were detected only by frg-ELISA, might also be important for *in vivo* protection. However that was not demonstrated in the present work. The hypothetical existence of protective frg-ELISA binding Abs could also explain why *in vitro* complement-dependent PNT are only detected in about 50% of VHSV survivors [15,16,18]. Fractionation of the sera by affinity columns using immobilized frgs and assay of the corresponding fractions by frg-ELISA, PNT and *in vivo* passive transfer, might be used in the future to investigate the possible functional significance of frg11-binding Abs.

Complement-dependent PNT Abs could be detected with the highest sensitivity when the homologous VHSV or very similar isolates were used for testing the sera from DK-211443 infected trout [9], despite their G protein sequences differing only by 24 amino acids with the DK-F1 VHSV isolate used for the PNT [9]. Similar results were obtained with some of the data reported here for farm A (Table 1). In comparison, frg-ELISA Abs could be detected in the sera from trout infected not only with the homologous VHSV-07.71 isolate but also with the heterologous VHSV-201433 isolate. The 13 amino acid difference between the VHSV-07.71 and DK-201443 isolates (2 mutations in frg11, 3 in frg15, 7 in frg16 and 11 in G21-465) had no apparent effect on the frg-ELISA

Table 1
Comparison among PNT, VHSV-ELISA and frg11-ELISA titres in rainbow trout sera obtained from 2 different located farms after VHSV outbreaks with high or medium mortalities.

Rainbow trout farm	Serum No.	50% PNT titre			ELISA titre	
		VHSV isolate used in test:			Solid-phase	
		F25	201433-40	Homologous	VHSV F25	frg11 07.71
A	1	<40	<40	<40	<40	0.08
	2	**1280**	**2560**	**5120**	**640**	−0.02
	3	<40	**1280**	**320**	<40	**0.27**
	4	<40	**640**	**160**	**2560**	−0.01
	5	**80**	<40	**80**	**2560**	0.00
	6	<40	<40	**80**	<40	**0.92**
	7	<40	<40	**2560**	**640**	0.01
	8	**5120**	**2560**	**5120**	**640**	**0.19**
	9	<40	**5120**	**5120**	**2560**	0.05
	10	<40	<40	**640**	<40	**0.20**
	11	<40	<40	**320**	<40	0.03
	12	<40	**2560**	<40	**640**	**0.23**
	13	<40	<40	<40	<40	0.02
	14	<40	<40	<40	**2560**	**0.17**
	15	<40	**5120**	**5120**	**2560**	0.08
	16	<40	<40	80	<40	**0.28**
	17	40	<40	320	**640**	**0.19**
	18	<40	**640**	**2560**	**160**	0.05
	19	<40	**640**	**1280**	**160**	−0.31
	20	<40	**2560**	**5120**	**640**	0.13
	Positives, %	**15**	**50**	**70**	**65**	**40**
B	1	–	–	<40	<40	0.03
	2	–	–	<40	<40	**0.26**
	3	–	–	<40	<40	**0.44**
	4	–	–	<40	<40	**0.28**
	5	–	–	<40	**160**	**0.35**
	6	–	–	<40	**160**	**0.47**
	7	–	–	<40	<40	0.16
	8	–	–	<40	<40	**0.20**
	9	–	–	<40	<40	**0.49**
	10	–	–	**320**	**640**	**0.24**
	11	–	–	<40	<40	−0.07
	12	–	–	<40	<40	**0.35**
	13	–	–	<40	<40	**0.63**
	14	–	–	<40	40	**0.28**
	15	–	–	<40	<40	**0.77**
	16	–	–	<40	<40	**0.49**
	17	–	–	**40**	<40	0.10
	18	–	–	**80**	<40	**0.37**
	19	–	–	<40	<40	**0.45**
	20	–	–	<40	<40	**0.18**
	Positives, %			**15**	**15**	**80**

Rainbow trout sera were collected from two farms in Denmark different locations after VHSV outbreaks with high (~80%) and medium (~50%) mortalities in farm A in 2006 (206277) and in farm B in 2008 (2008-50-362), respectively. ELISA was performed by either using captured-VHSV according to previously reported methods [9], frg11 or bovine serum albumin (BSA) as solid phases – not estimated. Homologous, the VHSV isolate used for the test was the one collected from the outbreak. The absorbance values obtained in plates coated with BSA were substracted from the absorbances values obtained with plates coated with frg11 and cut-offs calculated accordingly. To increase their sensitivity, cut-offs were chosen as <40 for PNT and VHSV-ELISA. For frg11-ELISA, mean plus 2 standard deviations of non-infected sera ($n = 15$) gave a cut-off value of 0.17. Numbers in bold, rainbow trout sera used as positives in each method to calculate percentage of positives (%).

performances. However, whether a similar effect might be present in frg-ELISA with other VHSV isolates is not known. Because the highest sequence variability on the protein G of VHSV was localized in amino acid 245–300 [2,4], new frg15 or frg16-like recombinants which will avoid the 245–300 hypervariable region, might be required to increase the scope of VHSV isolates which could be detected by those frgs. On the other hand, most of the variability found in frg11 was only restricted to 2 short amino acid stretches [29] and therefore frg11 might detect more isolates than frg15 or frg16. An extensive work with more VHSV isolates and/or with the corresponding different sequence variations in frgs, will have to be performed to demonstrate their ability to detect Abs in trout infected with any VHSV isolate.

A high correlation existed among the results obtained by frg-ELISA whether frg11, frg15, frg16 or G21-465 were used for the assays. Among the frgs used, frg11 (amino acids 56–110) had the lowest backgrounds and therefore did not required additional washing steps. Previous work showed that frg11 from VHSV-07.71, could be used in ELISA to detect trout Abs to VHSV-07.71 [28]. Frg11 was initially selected because ~ 40% of sera from trout hyperimmunised by repeated injections with purified VHSV-07.71 [8], recognized the pepscan 15-mer peptides defining frg11, a stretch related to VHSV fusion [23]. However, under the conditions reported before [28], a serum from a hyperimmunized rainbow trout could only be ~ 100–200-fold diluted before the ELISA absorbances reached background absorbances obtained in the absence of any trout sera. In contrast, under the optimized conditions described in this work, hyperimmunized trout sera could be diluted up more than 10,000 fold to reach background absorbances (Fig. 1). Because anti-VHSV trout Abs of the IgM class are usually of low affinity and bind non-specifically to almost any surface, they often produce false positives [25]. That phenomena have required the use of solid-phase Ab captured-VHSV in the presence and in the absence of VHSV to distinguish true positives [9,12,25] and caused also difficulties when using lectin-affinity purified protein G [26] and/or recombinant yeast G4 [6]. Frg-dependent backgrounds could be controlled in this work by blocking the frg-coated solid phases with low amounts of milk and by adding a final washing step of 0.5 M NaCl, but those additives had to be carefully adjusted in concentration to avoid reducing true signals. Further studies might be required to control the higher backgrounds expected with most of the frgs with trout sera from farms, since trout sera from controlled-laboratory infections, like those described above, tend to show lower cross-reactivities (data not shown). To preliminarily evaluate the potential use of frg11-ELISA to detect previous exposure to VHSV in natural infections, rainbow trout sera samples from 2 farms were obtained after having suffered their corresponding outbreaks and were analysed by several of the presently available methods (Table 1). To increase the specificity of the frg11-ELISA, the absorbance values obtained by coating the plates with BSA were subtracted from the absorbance values obtained with plates coated with frg11. Frg-ELISA resulted in higher prevalence of anti-VHSV Abs in sera from farm B (medium mortalities) than in the sera from farm A (higher mortalities). However, because of the small amount of sera ($n = 20$ for each farm) and the large amount of variables of natural VHSV outbreaks, many more data should be gathered and studied before we can interpret those data and reach definitive conclusions.

ELISA might be preferred for massive screenings of trout sera for anti-VHSV Abs, because any kind of ELISA seems to be a simpler method to perform than complement-dependent PNT for that purpose. Further advantages of frg-ELISA could be that only the percentage of positive sera by frg-ELISA increased with time after VHSV infection and that frg-ELISA might be less sensitive to VHSV isolate sequence variations than PNT. However, it will be very important to know what the presence of those frg-binding Abs could mean from a functional standpoint (ie: whether those new frg-binding Abs are or are not protective) and whether or not more VHSV isolates could be detected by using G frgs than PNT. A practical method to detect survivors of VHSV natural outbreaks will be most welcome to detect asymptomatic carriers. However, because the number of sera studied here were limited and mostly came from laboratory-controlledconditions, further studies including many more sera from survivors of natural VHSV outbreaks with different VHSV isolates would need to be studied until such applications could be evaluated for frg-ELISA. Nevertheless, an ELISA method to detect trout serum Abs to VHSV, such as the one

described here, could be useful to follow immunization attempts during vaccine development and/or to follow the kinetics of fish Ab response during VHSV infections [13].

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