ELSEVIER

Journal of Virological Methods 58 (1996) 1–6

Journal of Virological Methods

Fast neutralization/immunoperoxidase assay for viral haemorrhagic septicaemia with anti-nucleoprotein monoclonal antibody

G. Lorenzo, A. Estepa, J.M. Coll*

INIA, CISA-Valdeolmos, Departamento de Sanidad Animal, 28130 Madrid, Spain

Accepted 21 September 1995

Abstract

An enzyme-immunohistochemical procedure was employed to facilitate neutralization/diagnostic tests for viral haemorrhagic septicaemia virus (VHSV), a significant pathogen in trout farms throughout Europe. The method described can be used for trout or mice antibodies; increases speed (1 day), simplicity, and minimizes the use of reagents compared to other neutralization assays. Furthermore, the test requires a minimum handling of the cell cultures under sterile conditions, decreasing frequent contamination due to the non-sterile conditions of the fish pathological samples. Foci of 5–20 infected epithelioma papillosum carp (EPC) cells are detected and counted with an inverted microscope in under 16 h after infection of EPC monolayers using a high titre anti-N VHSV monoclonal antibody (MAb) 2C9. MAb 2C9 recognizes different viral haemorrhagic septicaemia virus serotypes and VHSV isolates from different host species (trout, salmon and barbel) and Spanish geographical locations. The high titre and specificity of MAb 2C9 favour its conjugation to peroxidase and also make it possible to use in direct immunoperoxidase staining of the VHSV infected EPC monolayers. This neutralization/immunoperoxidase assay should improve diagnostics that use currently agarose or methylcellulose plaque reduction neutralization assays.

Keywords: Viral haemorrhagic septicaemia virus; Trout antibodies; Enzyme-immunohistochemical procedure

1. Introduction

Current methods of fish rhabdovirus detection and identification are based on cell culture amplification and subsequent neutralization (Sanz and Coll, 1992a). These methods are time-consuming (5–6 days), labour-intensive and require well equipped laboratories mostly due to the need to work under sterile conditions. Other techniques, such as immunofluorescence, ELISA, or immunoblot, although useful in research, have limited application in diagnosis because of either low sensitivity, technical complexity, difficulty of interpretation or need for specialized equipment (McAllister and Schill, 1986; Mourton et al., 1990; Mourton et al., 1992; Olesen et al., 1991; Olesen et al., 1993; Olesen and Vestergard-Jorgensen, 1986; Sanz and Coll, 1992b), while new techniques based on PCR or ELISA with PCR

* Corresponding author.

SSDI 0166-0934(95)01972-3

(Estepa et al., 1995) have not yet been applied successfully to clinical diagnosis.

Rhabdoviruses cause significant annual losses in salmonid farms in Europe and North America (DeKinkelin, 1972). The neutralizing antibody to viral haemorrhagic septicaemia virus (VHSV), a rhabdovirus affecting trout, only reacts with the glycoprotein G (Lorenzen et al., 1988; Lorenzen et al., 1990) as in other rhabdoviruses (Coll, 1995a). Of the five virion proteins of VHSV, L (the RNA polymerase of ≈ 200 kDa), N/Nx (the nucleoprotein of 45–50 kDa), M_1/M_2 (the matrix proteins of 22–28 kDa) and G (the neutralizing epitope-carrier glycoprotein of 60–80 kDa) (Deuter and Enzmann, 1986; McAllister and Wagner, 1975), the N is not only the majority component of virions (Basurco and Coll, 1989) but is also the earliest to be synthesized after VHSV infection (Bernard and DeKinkelin, 1985). In the experiments reported now, a MAb (Coll and Dominguez, 1994) was selected from a panel of anti-N MAbs (Sanz et al., 1993; Sanz and Coll, 1992c) for use for immunoperoxidase staining of VHSV infected EPC monolayers for use in rapid (≤ 1 day) and for simpler neutralization diagnostic tests.

2. Materials and methods

2.1. Viruses

The viruses used to characterize the MAbs were VHSV-F_1, VHSV-F_2, and VSHV-23.75. VHSV Spanish isolates were isolated from rainbow trout, *Onchorynchus mykiss*, Richardson (689 from Galicia in 1984; 471 from Navarra in 1986; and 144 from Salamanca in 1984), Atlantic salmon, Salmo salar, Linneus (472, from Cantabria in 1986) and barbel, *Barbus graellsi*, Steindachner (798 from Aragón in 1986) (Basurco and Coll, 1989; Basurco and Coll, 1992). Epithelioma papillosum cyprini (EPC) cell culture techniques and virus purification were carried out essentially as reported by DeKinkelin (1972). Supernatants from VHSV 07.71 infected EPC, were clarified by centrifugation at 20 000 $\times$ *g* for 20 min, and kept frozen at -70°C in aliquots until used as inoculum in the neutralization assays.

2.2. Anti-G VHSV antibodies to neutralize VHSV

Anti-p2 was polyclonal mice ascites obtained by immunizing Balb-c with the peptide p2 (aa82–106) from the G protein of VHSV. The anti-p2 antibodies were purified by affinity chromatography over immobilized p2 at pH 7.6 as described by Estepa and Coll (1995). MAb C10 neutralizing VHSV was obtained from Sanofi Diagnostic Pasteur (Marnes-La-Coquette, France).

2.3. Anti-N VHSV monoclonal antibodies (MAbs) to detect VHSV infected EPC foci

Anti-N MAbs were obtained using the myeloma cell line P3X63-Ag8653 (Sanz et al., 1993). Mouse ascites from the hybridoma 2C9 were obtained by injection of physiological saline as described (Coll, 1989). The ascites, clarified by low speed centrifugation, were purified by affinity chromatography using Protein A-Sepharose columns (Pharmacia, Uppsala, Sweden) in sample buffer (1.5 M glycine, 3 M NaCl, pH 8.9). The retained fractions of MAb 2C9 (IgG2a) eluted with 0.1 M citric acid, pH 4.9, were pooled and dialyzed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.2 (PBS). Purity, tested by gel electrophoresis, was demonstrated by two single Coomassie-blue stained bands at 50 and 24 kDa, respectively. To couple peroxidase to MAb 2C9, 0.7 mg of MAb was mixed with 20 mg of horseradish peroxidase, E.C. 1.11.1.7., 1000 U/mg, RZ/3.3 (Boehringer Mannheim) in 0.4 ml of PBS pH 7.2 and 10 μl of 25% glutaraldehyde. After incubation at 37°C during 2 h, 400 μl of 1 M glycine were added to quench the glutaraldehyde.

2.4. Immunoperoxidase staining procedure

About 10^3 $TCID_{50}$ per ml of VHSV 07.71 were incubated overnight at 4°C with serial dilutions of anti-VHSV antibodies. Then, cultures of EPC cells in 96-well plates were infected with 100 μl of the different virus/antibody mixtures, adsorbed during 1 h at 14°C with agitation, washed with cell culture medium and filled with 100 μl/well of culture

medium. The VHSV infected EPC were incubated overnight at 14°C. To assay for trout antibodies, 10% of fresh trout serum was added to the virus/antibody mixtures as a source of trout complement. The VHSV infected EPC monolayers were fixed during 10 min in cold methanol and dried. To detect the N antigen, the MAb 2C9 diluted 1000-fold in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.24 mM merthiolate, 5 g of Tween 20/l, 50 mg of phenol red/l, pH 6.8) was added to the wells (100 μl/well) and incubated with agitation during 1 h. After washing with distilled water, 100 μl of peroxidase-labelled anti-mouse IgG (Nordic, Tilburg, The Netherlands) were added per well and incubation continued for 30 min. Alternatively, EPC cultures were stained with a 100-fold dilution of the 2C9-peroxidase conjugate (Sanz and Coll, 1992c). After three washings by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma, Che. Co) in the appropriate buffer was added, and the reaction was allowed to proceed until brown foci were detected by inspection with an inverted microscope in the controls containing no antibodies.

Once washed with water and air dried, brown foci (DAB positive foci) were counted using an inverted microscope provided with a 10 × ocular eye grid. The results were expressed by the following formula, number of DAB positive foci in the presence of MAb/number of DAB positive foci in the absence of NAb × 100. Neutralization titre was defined as the reciprocal of the maximal dilution that reduced DAB positive foci to a minimum number, usually 1–2 per well.

2.5. Plaque neutralization assays

Plaque neutralization assays were prepared in 24-well plates, with overlays of 0.8% ultra-low gelling temperature agarose (Sigma Chemical Co., St Louis, MO, U.S.A.). Alternatively, cultures were overlaid with 0.5% methylcellulose in cell culture medium. VHSV (200–300 μl) and serial dilutions of MAbs (100–200 μl) were mixed and incubated overnight at 14°C (Sanz and Coll, 1992d). The mixtures were then absorbed to EPC monolayers at 14°C during 1 h, washed, overlaid with 400 μl of 0.8% agarose or 0.5% methylcellulose per well and incubated at 14°C for 4–5 days. Visible plaques were counted and plaque forming units (PFU) results were expressed by the following formula, number of plaques in the presence of MAb/number of plaques in the absence of MAb × 100. The neutralization titre was defined as the reciprocal of the maximal dilution that reduced the VHSV plaques to a minimum number.

2.6. Production of trout survivors of VHSV infection

Outbred trout (0.5–2 g per trout) were infected for 2 h at 12–14°C with 10^6 $TCID_{50}$/ml of VHSV attenuated by 10 passages on EPC cells (Basurco and Coll, 1992). Under these conditions, trout survival was 10 to 30% ($n = 3$, n = number of experiments). The trout surviving the infection were challenged 2 months later with VHSV isolated on EPC cells from infected trout (10^6 $TCID_{50}$/ml, 2 h, 10–11°C). Between 5 to 24% ($n = 3$) of the initial number of trout survived both infections (DeKinkelin, 1988), showed no signs of VHS and used 4–8 months after the last VHSV challenge (Enzmann and Konrad, 1993). Control non-infected trout were maintained in parallel aquaria.

3. Results

Titres of purified anti-N MAbs were 1/5000 for 2D5 (IgG_1K), 1/6000 for 3E7 (IgG_1K) and 1/31 000 for 2C9 ($IgG_{2a}K$). Although the three anti-N MAbs (2D5, 2C9 and 3E7) reacted with 5 VHSV Spanish isolates and the 3 VHSV serotypes (F1, F2 and 23.75) only 2C9 was useful for direct immunoperoxidase techniques.

MAb 2C9 stained the VHSV 07.71 infected EPC monolayers by generating brown foci consisting of 5–20 cells using the direct or indirect immunoperoxidase technique (Fig. 1). The stained pattern obtained allowed easy identification and counting of foci forming units or DAB positive foci.

Fig. 2 shows the percentage of DAB positive foci obtained by the neutralization/immunoperoxidase assay with anti-p2 (Estepa and Coll, 1995) and with MAb C10 using either 1500 or 6000 plaque form-

ing units of VHSV per ml. The neutralization titre obtained for anti-p2 was non-significant for both plaque forming assays or/and the neutralization/immunoperoxidase assay. The neutralization titres obtained for C10 were similar (1000–3000) whether the plaque forming assay or the neutralization/immunoperoxidase assay were used (Fig. 2).

Fig. 3 shows the percentage of DAB positive foci obtained by the neutralization/immunoperoxidase assay with serum obtained from a population of trout whose blood was sampled at different times (from 0 to 8 months) after surviving two VHSV infections. Neutralization titres varied from 0 in the initial non-infected trout (n = 3), to between 100 and 500, 4–6 months after the last VHSV infection (n = 3) and to non-detectable, 8 months after the last VHSV infection (n = 3).

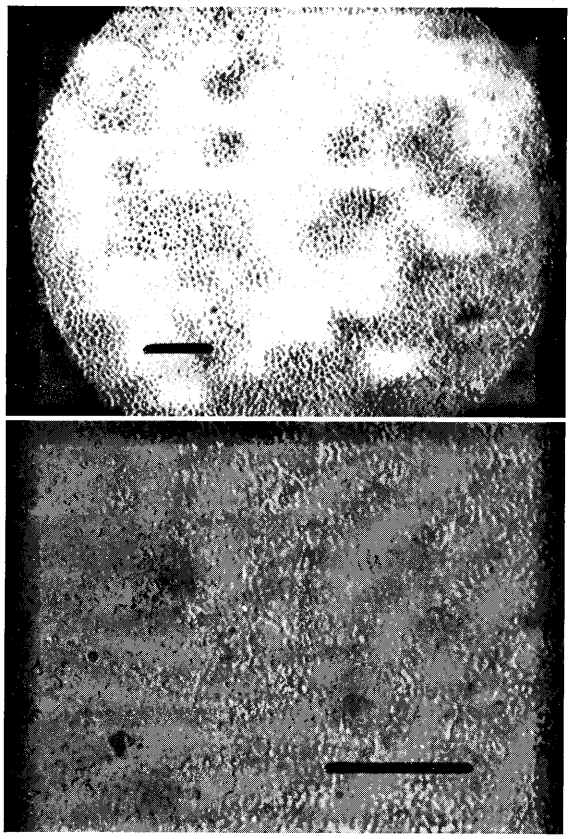

Fig. 1. VHSV-infected EPC cell monolayer stained by immunoperoxidase at low (up) and high (low) magnifications. EPC monolayers in 96-well plates (containing about 300 000 EPC cells/well) were incubated at 14°C overnight with 600 PFU of VHSV 07.71/well. Monolayers were fixed and stained with MAb 2C9 and DAB as indicated. Bars are 100 μ.

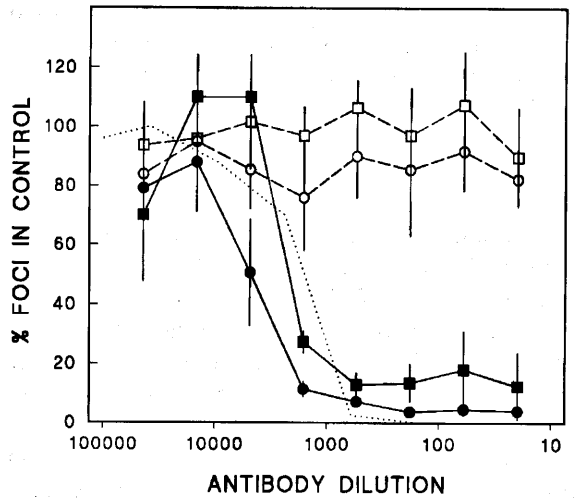

Fig. 2. Percentage of DAB-positive foci obtained by the neutralization/immunoperoxidase assay with anti-p2 and MAb C10. Controls of VHSV 07.71 were included in each of the experiments. Means and standard deviations of duplicate counts are represented. ●, C10 neutralization of 6000 PFU/ml; ■, C10 neutralization of 1500 PFU/ml; ○, anti-p2 neutralization of 6000 PFU/ml; – □ –, anti-p2 neutralization of 1500 PFU/ml. · · ·, C10 percentage of plaques obtained by the plaque assay using methylcellulose (included for comparison).

4. Discussion

MAbs anti-N were selected to optimize the neutralization/immunoperoxidase assay for VHSV because the N protein is probably one of the least variable of the rhabdoviruses in general (Bernard et al., 1990; Bernard et al., 1991; Bernard et al., 1992) and this assay could therefore be used for most viral serotypes and isolates. Furthermore, the N protein of VHS is a major component found both in the complete virus and in infected cells (McAllister and Wagner, 1975) and the N protein is the first VHSV protein to be synthesized 1–3 h after infection (Bernard and DeKinkelin, 1985). On the other hand, the N

epitopes defined by MAb 2C9 were highly conserved in three different VHSV serotypes and in several Spanish isolates (Basurco and Coll, 1989).

Different multiplicities of infection could be used with similar results (Fig. 2) and the titre obtained for C10 compares well with the titer obtained by visual plaque reduction assays and with commercially available documentation (equivalent titre of 1250). The assay can also be adapted to trout serum. In this study VHSV neutralization titres of serum were determined from VHSV-infected trout survivors (Lorenzo et al., 1995) and obtained similar results to those reported before using visual plaque reduction assays (Enzmann and Konrad, 1993).

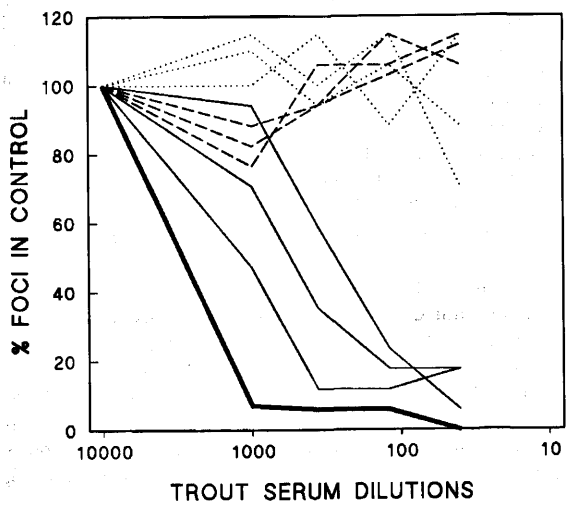

Fig. 3. Percentage of DAB-positive foci obtained by the neutralizing/immunoperoxidase assay of trout serum from fish surviving two VHSV infections. Serial dilutions (3-fold) of trout serum were incubated with 1000 PFU of VHSV in the presence of 10% fresh trout serum (source of complement) at 4°C overnight. Next day, mixtures were added to EPC monolayers in 96-well plates, adsorbed over 1 h at 4°C, washed and fill with 100 μl/well of cell culture medium. After overnight incubation at 14°C, the cultures were fixed with cold methanol during 10 min and dried. Staining of the VHSV foci was with MAb 2C9, peroxidase labelled rabbit anti-mouse IgG and diamino-benzidine (DAB). · · ·, negative control serum from three non-infected trout; ——, serum from three trout surviving 4–6 months after the VHSV infections; - - -, serum from three trout surviving 8 months after the VHSV infections; ——, 5-fold diluted serum from a surviving trout with very high neutralization titre (gift of Dr. DeKinkelin).

The immunoperoxidase technique provides some advantages over the use of other labels, such as immunofluorescence to detect VHSV foci, i.e. there is no background staining, an ordinary light microscope can be used and results can be stored for a long time. Due to their high titre, MAb 2C9 can also be useful for a simpler direct immunoperoxidase assay. Samples from VHSV infections can be assayed with a high degree of specificity and over a short period of time (less than 1 day) after infection of EPC cell culture monolayers using this method.

Acknowledgements

We thank Dr. DeKinkelin (INRA, Jouy-en-Josas, France) for the gift of trout serum with a high VHSV neutralization titer and the VHSV-23.75. Thanks are also due to the late Dr. Vestergaard-Jorgensen for the VHSV F1 and F2 and to whose memory this work is dedicated. We appreciated the help of C. Cooper in correcting the style and of J.P. Coll in typing the manuscript. This work was supported by Research Grants from the AIR2 Programme of the European Community, project CT94-1334, from the INIA project SC94-102, Spain and from CICYT project AGF95-910, Spain.

References

Basurco, B. and Coll, J.M. (1989) Spanish isolates and reference strains of viral haemorrhagic septicaemia virus shown similar protein size patterns. Bull. Eur. Assoc. Fish Pathol. 9, 92–95.

Basurco, B. and Coll, J.M. (1992) In vitro and in vivo immunization with the first viral haemorrhagic septicaemia viruses isolated in Spain compared to international reference serotypes. Res. Vet. Sci. 53, 93–97.

Bernard, J., Bremont, M. and Winton, J. (1991) Sequence homologies between the N genes of the 07.71 and Makah isolates of viral, haemorrhagic septicaemia virus. Proceedings of the II International Symposium on Viruses of Lower Vertebrates, Oregon State University, pp. 109–116.

Bernard, J., Bremont, M. and Winton, J. (1992) Nucleocapsid gene sequence of a North American isolate of viral haemorrhagic septicaemia virus, a fish rhabdovirus. J. Gen. Virol. 73, 1011–1014.

Bernard, J. and DeKinkelin, P. (1985) Effect of UV irradiation of Viral Haemorrhagic Septicemia Virus on virus-specific intracellular synthesis. Ann. Inst. Pasteur/Virology 136, 213–222.

Bernard, J., Lecocq-Xhonneux, F., Rossius, M., Thiry, M.E. and De Kinkelin, P. (1990) Cloning and sequencing the messenger RNA of the N gene of viral haemorrhagic septicaemia virus. J. Gen. Virol. 71, 1669–1674.

Coll, J.M. (1989) Injection of physiological saline facilitates recovery of ascitic fluids for antibody production. J. Immunol. Methods 104, 219–222.

Coll, J.M. (1995a) The glycoprotein of rhabdoviruses. Arch. Virol. 140, 827–851.

Coll, J.M. and Dominguez, J. (1994) Applications of monoclonal antibodies in Aquaculture. Biotechnol. Adv. 13, 45–73.

DeKinkelin, P. (1972) Le virus D'Egtved. II. Purification. Ann. Rech. Vét. 3, 199–208.

DeKinkelin, P. (1988) Vaccination against viral haemorrhagic septicaemia. In: A.E. Ellis (Ed.), Fish Vaccination, Academic Press, New York, pp. 172–192.

Deuter, A. and Enzmann, P.J. (1986) Comparative biochemical and serological studies on two fish-pathogenic rhabdoviruses (VHS-V and SVC-V). J. Vet. Med. 33, 36–46.

Enzmann, H. and Konrad, M. (1993) Longevity of antibodies in brown trout and rainbow trout following experimental infection with VHS virus. Bull. Eur. Assoc. Fish Pathol. 13, 193–194.

Estepa, A. and Coll, J.M. (1995) Pepscan mapping and fusion-related properties of the major phosphatidylserine binding domain of the glycoprotein of VHSV, a salmonid rhabdovirus. Virology (in press).

Estepa, A., DeBlas, C., Ponz, F. and Coll, J.M. (1995) Detection of trout haemorrhagic septicaemia rhabdovirus by capture with monoclonal antibodies and amplification with PCR. Bull. Eur. Assoc. Fish Pathol. 15, 49–53.

Lorenzen, N., Olesen, N.J. and Jorgensen, P.E.V. (1988) Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. Dis. Aquat. Org. 4, 35–42.

Lorenzen, N., Olesen, N.J. and Vestergaard-Jorgensen, P.E. (1990) Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. J. Gen. Virol. 71, 561–567.

Lorenzo, G.A., Estepa, A., Chylmonczyk, S. and Coll, J.M. (1995) Different peptides from hemorrhagic septicemia rhabdoviral protein stimulate leucocyte proliferation with individual fish variation. Virology 212 (in press).

McAllister, P.E. and Schill, W.B. (1986) Immunoblot assay: a rapid and sensitive method for identification of salmonid fish viruses. J. Wildl. Dis. 22, 468–474.

McAllister, P.E. and Wagner, R. (1975) Structural proteins of two salmonid rhabdoviruses. J. Virol. 15, 733–738.

Mourton, C., Bearzotti, M., Piechaczyk, M., Paolucci, F., Pau, B., Bastide, J.M. and Kinkelin, P.D. (1990) Antigen-capture ELISA for viral haemorrhagic septicaemia virus serotype I. J. Virol. Methods 29, 325–334.

Mourton, C., Romestand, B., DeKinkelin, P., Jeffroy, J., Le Gouvello, R. and Pau, B. (1992). Highly sensitive immunoassay for direct diagnosis of viral haemorrhagic septicaemia which uses antinucleocapsid monoclonal antibodies. J. Clin. Microbiol. 30, 2338–2345.

Olesen, N.J. Lorenzen, N. and Vestergaard-Jorgensen, P.E. (1991) Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). Dis. Aquat. Org. 10, 31–38.

Olesen, N.J., Lorenzen, N. and Jorgensen, P.E.V. (1993) Serological differences among isolates of viral haemorrhagic septicaemia virus detected by neutralizing monoclonal and polyclonal antibodies. Dis. Aquat. Org. 16, 163–170.

Olesen, N.J. and Vestergard-Jorgensen, P.E. (1986) Detection of neutralizing antibody to Egtved virus in Rainbow trout (*Salmo gairdneri*) by plaque neutralization test with complement addition. J. Appl. Icthyol. 2, 33–41.

Sanz, F., Basurco, B., Babin, M., Dominguez, J. and Coll, J.M. (1993) Monoclonal antibodies against the structural proteins of viral haemorrhagic septicaemia virus isolates. J. Fish Dis. 16, 53–63.

Sanz, F. and Coll, J.M. (1992a) Techniques for the diagnosis of the viral diseases of salmonids. Dis. Aquat. Org. 13, 211–223.

Sanz, F.A. and Coll, J.M. (1992b) Detection of hemorrhagic virus of salmonid fishes by use of an enzyme-linked immunosorbent assay containing high sodium chloride concentration and two concompetitive monoclonal antibodies against early viral nucleoproteins. Am. J. Vet. Res. 53, 897–903.

Sanz, F. and Coll, J.M. (1992c) Detection of viral haemorrhagic septicemia virus by direct immunoperoxidase with selected anti-nucleoprotein monoclonal antibody. Bull. Eur. Assoc. Fish Pathol. 12, 116–119.

Sanz, F. and Coll, J.M. (1992d) Neutralizing-enhancing monoclonal antibody recognizes the denatured glycoprotein of viral haemorrhagic septicaemia virus. Arch. Virol. 127, 223–232.