Detection of hemorrhagic septicemia virus of salmonid fishes by use of an enzyme-linked immunosorbent assay containing high sodium chloride concentration and two noncompetitive monoclonal antibodies against early viral nucleoproteins

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SUMMARY

Inclusion of high-ionic strength buffers helped us to develop a sandwich ELISA to detect hemorrhagic septicemia virus (HSV) in cell culture and infected trout tissue extracts. For maximal sensitivity of 0.1 to 0.2 ng/well/100 μl or about 10 to 50 $TCID_{50}$/well/100 μl, trout extracts were diluted 1:1 and assayed for the earliest synthesized nucleoprotein N. Simultaneous binding of the N protein from HSV in the sample to the wells coated with monoclonal antibody (2D5 against the N protein) and to the peroxidase-labeled monoclonal antibody (2C9 against the N protein) proceeded during a 2-hour incubation at 20 to 22 C (room temperature). The response was linear between 6 to 60 ng/well of purified virus. Monoclonal antibodies used were noncompetitive with each other and reacted with F_1, F_2, 23.75, and 5 Spanish isolates of HSV, but not with infectious hematopoietic necrosis or infectious pancreatic necrosis viruses. Tissue specimens with low content of HSV virus may now be assayed directly without use of cell culture, rapidly, and with high precision, during the acute phase of the disease in salmonid fishes.

The causative agent of hemorrhagic septicemia is a membrane-enclosed, negative strand RNA virus that buds from the infected cell membrane.[1] The disease affects salmonid fishes, causing severe damage in fish farms in Europe.[2-4]

The 5 agreed-on virion proteins of the rhabdovirus are designated by the letters, L (the RNA-dependent RNA polymerase of 150 to 200 kDa), N/Nx (the major phosphorylated nucleoproteins of 45 to 50 kDa),[5-7] M_1 and M_2 (the matrix proteins of 22 to 28 kDa) and G (the neutralizing epitope-carrier glycoprotein of the spikes [60 to 80 kDa]).

Current methods of diagnosis are based on cell culture and serum neutralization testing. These test are time-consuming and labor-intensive, and in addition, they only can be used in commercial laboratories. Other serologic techniques used for hemorrhagic septicemia virus (HSV) detection and identification, such as immunofluorescence (the technique used for routine diagnosis of rabies[8]), ELISA, or immunoblot analysis, have limited application because of low sensitivity, technical complexity, difficulty of interpretation, or need for specialized equipment.[9,10] It has been suggested that monoclonal antibodies (MAB) could improve sensitivity of immunofluorescence, ELISA or both.[11] Even though MAB against HSV and rhabdoviruses, in general, have been obtained and described,[12-15] few reports of the use of MAB for ELISA exist, most probably because of low sensitivity.[16] Major advantages of ELISA are the possibility of rapidly processing large numbers of samples, using simple equipment,[17,18] and easy and reproducible scale up.[19] A highly sensitive ELISA that could detect all the serotypes of HSV would be desirable.

The objective of the study reported here was to develop an immunoenzymatic method for detection of HSV in microtitration plate wells, using a sandwich ELISA containing 2 noncompetitive MAB against the earliest synthesized major protein of the virus (nucleoprotein N) in the presence of high NaCl concentration. This new ELISA has higher sensitivity than those based on polyclonal anti-bodies,[10,11] and detects all serotypes of HSV not F_1 only.[16]

Materials and Methods

Viruses—Strains of virus used were HSV-F_1[a] and HSV-F_2,[a] HSV-23.75[b] and infectious hematopoietic necrosis (IHN; Cedar strain).[c] Five HSV isolates[2] were obtained from fish tissue specimens in Spain; viruses were isolated from rainbow trout: *Onchorynchus mykiss,* Walbaum (689, from Galicia in 1984; 471, from Navarra in 1986; and 144, from Salamanca in 1984), *Salmo salar* Linneus (472, from Cantabria in 1986), and *Barbus graellsi,* Steindachner (798, from Aragón in 1986), as described by Basurco and Coll.[3,4] Unless otherwise indicated, isolate 144 was used throughout the study.

Cells, media, and virus purification—Epithelioma papillosum cyprini (EPC) cells were used throughout the study. Cell culture techniques (RPMI 1640 medium[d]) and virus purification were essentially as reported by De Kinkelin[1] and modified by Basurco and Coll.[3,4] Protein content of

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[b] Provided by Dr. P. De Kinkelin, INRA, Jouy en Josas, France.

[c] Provided by Dr. R. Hedrick, University of California, Davis.

[d] Flow Laboratories, Ayrshire, Scotland.

purified virus was estimated by binding of Coomassie blue G-250 dye, using bovine serum albumin as the standard[e] or by staining the viral protein bands separated by gel electrophoresis[20] with Coomassie blue and comparing them with standards. Protein concentration was also determined by measuring absorption at 280 nm, using an extinction coefficient of 1.4. Virus purity, as calculated from scan of Coomassie blue-stained electrophoresed proteins, was about 95%.[3]

Preparation of buffer solutions for ELISA—Dilution buffer contained 130 m*M* NaCl, 2 m*M* KCl, 8 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, 0.24 m*M* merthiolate, 5 g of Tween 20/L, and 50 mg of phenol red/L, pH 6.8. In later experiments, NaCl was added to this dilution buffer. The described buffer was used as the washing buffer, diluted tenfold with distilled water.

Monoclonal antibodies—The MAB against HSV and their recognition of serotypes and Spanish isolates have been produced and characterized. Briefly, female mice (Balb/C) were given 9 injections of 25 μg of concentrated virus over a period of 9 months, their spleen cells were fused with the myeloma cell line P3-X63-Ag8653, then were selected, cloned, and screened by use of indirect ELISA. Isotype was determined by ELISA, and specificity was determined by immunoblot analysis following described procedures.[18,21,22]

Acquisition of ascites fluid and purification of the MAB—Ascites fluid was obtained by inoculation of physiologic saline solution as described.[23] Ascites fluids were clarified by centrifugation at 3,000 x g for 10 minutes and stored at −40 C until use. Then, fluids were purified by use of affinity chromatography over protein A-Sepharose[f] columns. Sample buffer was 1.5*M* glycine-3*M* NaCl, pH 8.9. The retained IgG_1 was eluted with 0.1*M* citric acid, pH 5.8, and IgG_{2a} was eluted with 0.1*M* citric acid pH 4.9. Eluted fractions were pooled and dialized against phosphate-buffered saline solutions (PBSS; 10 m*M* sodium phosphate-150 m*M* NaCl, pH 7.2). Purity, as tested by gel electrophoresis gave 2 single Coomassie blue-stained bands at 50 and 24 kDa, respectively. The MAB protein concentration was determined by measuring absorption at 280 nm, using an extinction coefficient of 1.4.

Indirect ELISA—The MAB and their conjugates were titrated by ELISA on HSV-coated microtitration plate wells. Briefly, 1 μg of purified HSV was added to the wells of polystyrene plates in 100 μl of distilled water, dried overnight at 37 C, then washed with washing buffer. The MAB were diluted in dilution buffer and 100 μl was added to the wells. After 1 hour's incubation and washing, 100 μl of peroxidase-labeled anti-mouse IgG[g] or MAB was added, and incubation continued for 30 minutes until color development. Titer was defined as the dilution of the MAB that gave absorbance ($A_{492\text{-}620\ nm}$) of 0.3 over background, ≤ 0.1.

Peroxidase conjugates—To couple peroxidase to MAB by use of one-step glutaraldehyde method, 0.7 mg of HSV antibodies was mixed with 10 mg of horseradish peroxidase (EC 1.11.1.7., 1,000 U/mg)[h] in 0.4 ml of PBSS, pH 7.2 and 10 μl of 25% glutaraldehyde. After incubation at 37 C for 1 hour, 400 μl of 1*M* glycine was added, and incubation was overnight at room temperature. The resulting conjugates had a molar ratio of antibody to peroxidase of 0.9:1.[24]

Sandwich ELISA—A sandwich ELISA was developed for capture and detection of HSV, following essentially described methods.[18,25] Polystyrene plates[i] were coated with 1 μg of MAB 2D5/100 μl of distilled water/well, dried overnight at 37 C and blocked by washing with tenfold-diluted dilution buffer. After this step, plates were dried for 2 hours at 37 C and kept sealed in the presence of silica gel at 4 C.

The 2-step assay was performed as follows. Fifty microliters of fish homogenates was pipetted into the wells, then 50 μl of dilution buffer was added. Plates were incubated for 60 minutes at room temperature, then were washed with washing buffer. Then, 100 μl of anti-VHS virus MAB 2C9 coupled to peroxidase (diluted to 0.2 μg/well in dilution buffer) was added, and plates were incubated 60 minutes at room temperature then washed 4 times with washing buffer.

The 1-step assay was as follows. Fifty microliters of fish homogenates was pipetted into the wells, followed by 50 μl of conjugate (diluted to 0.4 μg/well) in dilution buffer containing 1*M* NaCl, then wells were incubated for 120 minutes at room temperature. After incubation, plates were washed 4 times with washing buffer.

For the 1- and 2-step assays, 50 μl of substrate buffer (150 m*M* sodium citrate, 0.24 m*M* merthiolate, 3 m*M* H_2O_2 and 1 mg of o-phenylenediamine/L, pH 4.8)[26] was added, and the reaction was stopped by addition of 50 μl of 4*M* H_2SO_4 after 30 minutes. Results were read[j] at 3 wavelengths—450, 492, and 620 nm. Absorbance at 620 nm was used to correct for individual nonsignificant differences between wells. Absorbance at 450 was used to calculate the 492-nm absorbance values ≥ 2 by approximation (absorbance at 492 nm = absorbance at 450 nm × 2.4). Sensitivity was calculated for a 95% confidence level by assuming normal distribution of background values, as suggested by Tijssen.[27]

Inoculation of trout fingerlings with HSV—Trout (*Oncorhynchus mykiss*) were purchased from commercial farms after several annual tests indicated they were free of infectious pancreatic necrosis (IPN) virus.[2] Trout (0.2 to 1 g) were kept in 30-L aquariums with dechlorinated free-flowing water at 10 to 14 C. To infect with HSV, the water level of the aquarium was decreased to 2 L, then cooled to 8 to 10 C, virus was added (10^6 $TCID_{50}$/ml), and the trout were subjected to strong aeration over 2 hours. Water flow was restored to 1 L/min, and temperature was maintained at 10 to 11 C. The virus used was Spanish isolate 144[3,4] recovered from trout, amplified twice by culture in EPC, and used fresh. Dead fish were removed from each tank and weighed. Number of deaths was recorded daily. Dead fish were frozen at −40 C and processed for virus isolation as reported.[2] Heads and tails were removed, and

[e] Bio-Rad Laboratories, Richmond, Va.
[f] Pharmacia, Uppsala, Sweden.
[g] Nordic, Tilburg, The Netherlands.
[h] RZ/3.3, Boehringer-Mannheinn, Penzberg, Germany.
[i] Dynatech, Plochingen, Germany.
[j] Titertek Multiskan, Richmond, Va.

the viscera (about 0.4 g/trout) were cut with scissors and homogenized in a Potter-Elvehjem homogenizer (a glass tube provided with a pestle) after adding 2 ml of cell culture medium/trout. The homogenates were centrifuged at 10,000 × g for 10 minutes and supernatants were frozen at −40 C until assayed. Mean ± SD protein concentration was 48 ± 43 mg/ml (n = 34). Trout were processed individually or pooled in groups of 3.

Results

Selection of the MAB against nucleoprotein N—Three MAB against N were selected from a panel of 19 HSV MAB by results of immunoblot analysis. The 3 MAB were obtained as mouse ascites fluid, purified by affinity chromatography over protein-A and titrated by indirect ELISA. Titer was 1:5,000 for 2D5, 1:6,000 for 3E7, and 1:31,000 for 2C9. Peroxidase conjugates optimized by addition of glutaraldehyde for various durations gave titer of 1:4,200 for 2D5, 1:170 for 3E7, and 1:1,500 for 2C9. By using MAB 2D5 for coating the solid phase and MAB 2C9 as conjugate, absorbance values proportional to the concentration of HSV could be obtained. Coating the solid phase with the other MAB or with M_2 antibody resulted in 0 absorbance. Reaction of avidin-peroxidase to biotinylated MAB 2C9 did not improve the titer significatively (data not shown).

Before adding the MAB 2C9 conjugate, the HSV captured by the solid-phase MAB 2D5 was allowed to react with other MAB. Monoclonal antibody 2C9 was able to compete for binding with the MAB 2C9 conjugate, but not with any of the other MAB (Fig 1).

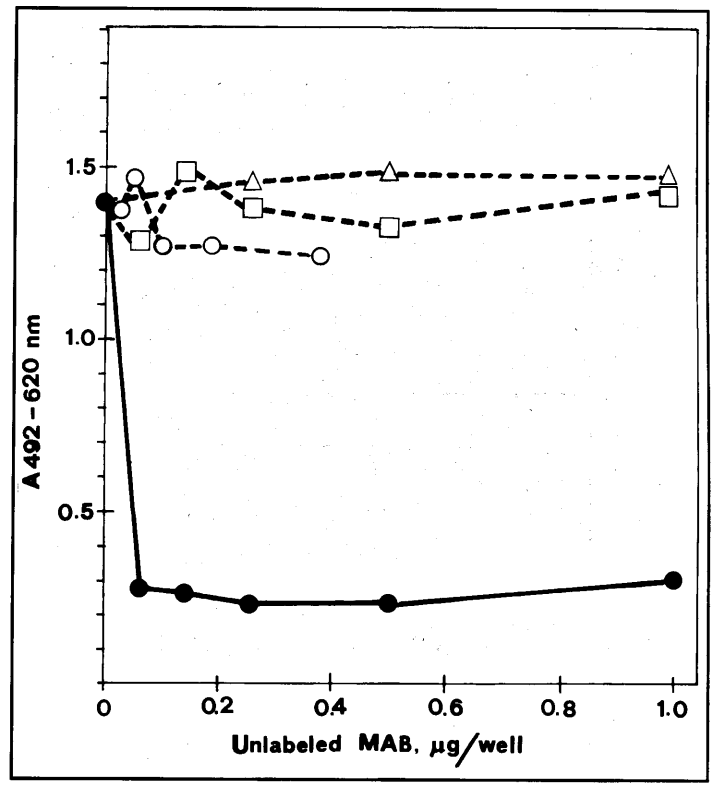

Figure 1—Competition between monoclonal antibody (MAB) 2C9 and other MAB against hemorrhagic septicemia virus (HSV). Unlabeled MAB were added to HSV (1 µg/well) captured by solid-phase MAB 2D5 (1 µg/well) before adding the peroxidase-labeled MAB 2C9 (0.2 µg/well). The ELISA was performed, using 1*M* NaCl and the 2-step assay. ●-●, MAB 2C9 (anti-N); ○—○, MAB 2D5 (anti-N); □—□, MAB 3E7 (anti-N); △—△, MAB 1H10 (anti-G).

Improvement of sensitivity—Even though MAB 2D5 and 2C9 were able to capture and detect HSV in a 2-step assay, the sensitivity of the assay was only about 1,000 ng/well. Addition of NaCl to the dilution buffer, increased the sensitivity to about 20 ng/well. The minimal concentration of NaCl that had to be added to the dilution buffer was 1*M*. Higher concentration did not increase sensitivity.

Simultaneous addition to the wells and incubation of virus and conjugate (the so-called 1-step assay) further increased sensitivity to about 0.2 ng/well (Fig 2). Neither order of addition nor previous mixing of the virus and conjugate had further effect on sensitivity. Optimal incubation time was 2 hours at room temperature. Increase of absorbance was linear up to 2 hours, then plateaued (not shown).

Optimal conditions for the ELISA were obtained after testing inclusion of 1% Triton X100 in the dilution buffer, several pH values (from 5 to 8) several conjugate incubation durations, coating of solid phase at 4 C in humidified chamber or at 37 C under dry conditions overnight, various bovine serum albumin batches, coating of solid phase by use of glutaraldehyde, use of 2 MAB for coating the solid phase, and use of polyclonal anti-mouse IgG to capture the coating MAB. None of these variations resulted in improvement of sensitivity.

Solid phases tested included 96-well microtitration plates[i] and microtitration plates divided in rows of 8 x 2 wells[k] of medium binding capacity. Results were similar in each instance. Coated dried solid phases were stable for at least 1 month at 4 C.

Analytic variables—The linear part of the 1-step assay was between 0.5 to 3.5 absorbance units at 492 to 620 nm, corresponding to 6 to 60 ng of virus/well (Fig 3). Hook effect was not found, up to maximal concentration of 100 ng of virus/well. Mean background absorbance values (in the absence of added virus) varied between experiments from 0.055 to 0.085 ± 18.8% (n = 4). Sensitivity, defined as the lowest virus concentration that can be measured over the background with 95% confidence by assuming normal distribution, varied. Therefore, sensitivity ranged between absorbances of 0.07 and 0.11, or 0.1 to 0.2 ng of virus/well, or 10 to 50 apparent $TCID_{50}$/well.

Intra-assay coefficient of variation for absorbance of the standards varied between 3 and 6% (Fig 3). Intra-assay coefficient of variation among independent duplicate samples of low, medium, and high virus concentrations (between 0 to 4,000 apparent $TCID_{50}$/well) varied between 3 and 23.5% (Table 2).

Specificity—The MAB selected were evaluated by ELISA performed by coating 0.3 µg of purified viral protein/well, followed by addition of peroxidase-labeled rabbit anti-mouse immunoglobulins. Five Spanish HSV isolates and F_1, F_2, and 23.75 HSV serotypes were used to coat the wells. The supernatants of the hybridomas were diluted 20-fold in dilution buffer to yield ELISA titer of about the

[k] Microwell model F-16, Nunc, Kaamstrup, Denmark.

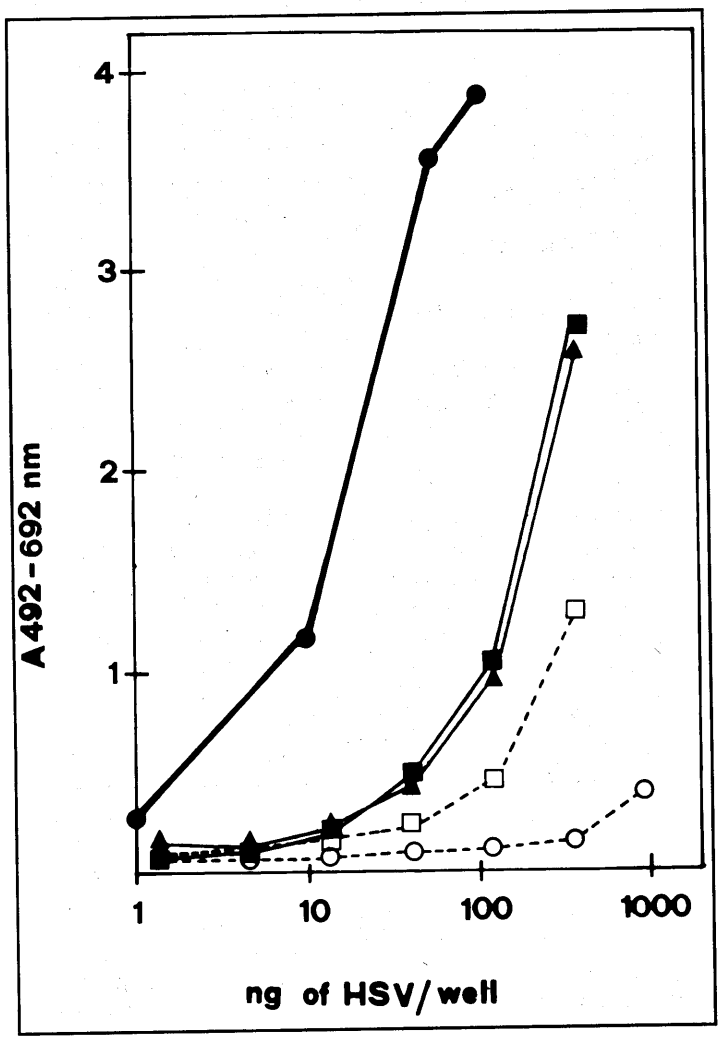

Figure 2—Enhancement of sensitivity by use of double concentration of conjugate, increased NaCl concentration, and the 1-step assay. All results were obtained, using MAB 2C9 labeled with peroxidase at 0.4 μg/well, and the dilution buffer defined in Materials and Methods. Sodium chloride was added to the dilution buffer at the indicated final concentrations. ●—●, 1*M* NaCl, 1-step assay; ■—■, 2*M* NaCl, 2-step assay; ▲—▲, 1*M* NaCl, 2-step assay; □—□, 0.5*M* NaCl, 2-step assay; ○—○, 0.13*M* NaCl, 2-step assay.

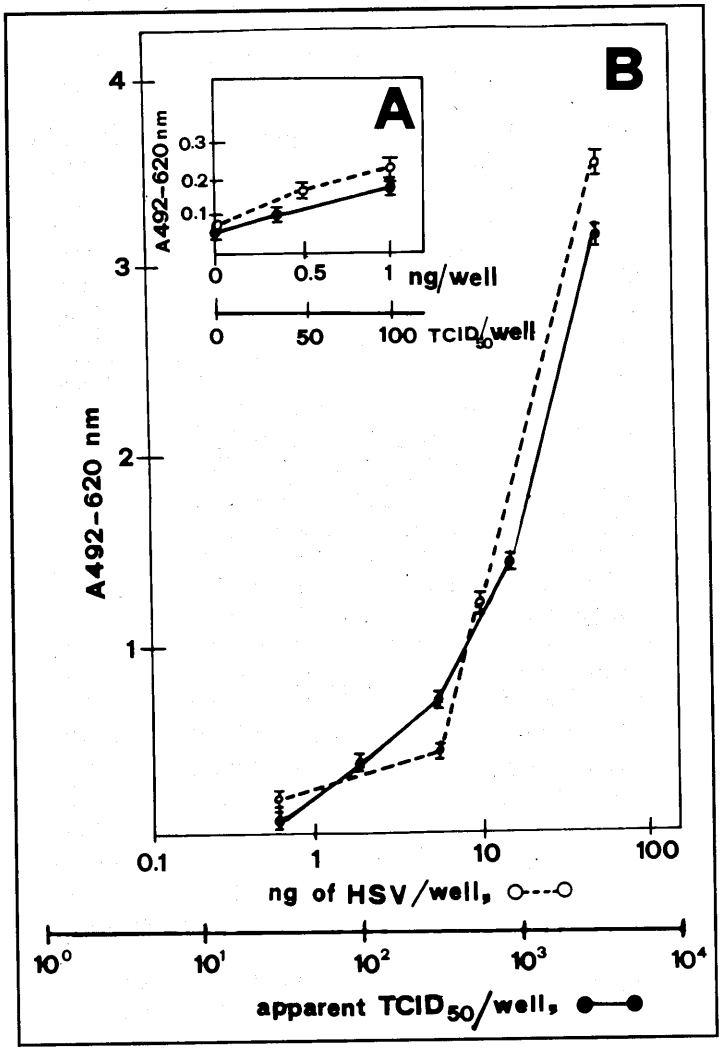

Figure 3—Typical standard curves for HSV detection by the use of ELISA. A–Low concentrations plotted on a double linear scale. B–Results, using a semilogarithmic scale. Points are mean ± SD $TCID_{50}$/well of 5 single measurements. ●—●, ng of purified virus; ○—○, supernatant from HSV-infected epithelioma papillosum cyprini (EPC) cells.

Table 1—Characteristics of monoclonal antibodies (MAB) selected for the sandwich ELISA

Use	Clone	Antigen	Isotype	Titer* ELISA	Fluorescence	Immunblot analysis‡
Solid phase	2D5	HSV-144†	G_1, K	5.0×10^3	+	N/Nx
Conjugate	2C9	HSV-144	G_{2a}, K	3.1×10^4	+	N/Nx

* Titer was obtained by ELISA, using 1 μg of purified virus/well; MAB were purified from the ascites induced by the hybridomas in mice. † Hemorraghic septicemia virus (HSV) was isolated (isolate 144) in Spain.[3,7] ‡ Specificity was defined by results of immunoblot analysis. Monoclonal antibodies 2D5 and 2C9 reacted with nucleoproteins N and Nx.

same order of magnitude. Results were normalized by the ELISA absorbances produced in parallel by use of rabbit polyvalent HSV antisera (F_1 + F_2 + 23.75) an international reference standard.[b] Among the 8 isolates tested, the coefficient of variation for the absorbance values was 7.1% for MAB 2D5 and was 7.2% for MAB 2C9.

Further experiments indicated that the sandwich assay recognized HSV F_1, F_2, and 23.75 nearly to the same extent, whereas neither IHN nor IPN virus was recognized (Fig 4).

Apparent $TCID_{50}$ in fish tissue homogenates—To define the cut-off between specimens with negative and positive results, homogenates of pooled healthy fish were prepared and assayed by use of the ELISA. Values < 122 to 186 $TCID_{50}$/well could not be considered positive results in adult fish, but in trout fingerlings, values of 17 $TCID_{50}$/well had to be considered positive results according to these data (Table 3). Healthy trout fingerlings had the lowest absorbance above background, compared with values for adults; thus, sensitivity for fingerlings was higher. When these data were transformed to apparent $TCID_{50}$/g of trout, values from 0 to 1333 were found (Fig 5). After the addition of purified virus to fingerling homogenates the ELISA value of HSV was 43.0 ± 9.9% (n = 3) of the initial value.

Twenty days after trout fingerlings were bath-infected with HSV, mortality was 77.6%. Dead trout were collected daily, frozen at −20 C, and all were homogenized, then

Table 2—Precision profile of apparent $TCID_{50}$ for fingerling trout homogenates

Concentration group ($TCID_{50}$/well)	n	Group mean (CV, %)* ($TCID_{50}$/well)
0 to 20	11	4 (11.9)
20 to 200	22	130 (3.0)
200 to 500	7	340 (23.5)
500 to 1000	14	700 (18.5)
1000 to 4000	15	1,650 (18.1)

* Mean and coefficient of variation (CV) of independent duplicates for each group of sera.

A total of 69 fingerling homogenates were analyzed in duplicate in 3 assays, and the results were grouped by apparent $TCID_{50}$/well.

Table 3—The ELISA absorbance values above background for the homogenates from several fish species

Species	Age	n	Sensitivity ($TCID_{50}$/well)
Salmo salar, Linnaeus	Adult	2	167
Barbus barbus, Steindachner	Adult	3	122
Sparus aurata, Linnaeus	Adult	8	186
Onchorynchus mykiss	Fingerlings	8	17
	Adult	22	142

Each n value was the result of a homogenate either from 5 pooled pronephros in the case of adults or 5 pooled fingerlings after removal of their head and tail. Sensitivty was defined as the lowest $TCID_{50}$ that could be detected over the background with 95% confidence, assuming normal distribution (sensitivity = mean + 2 SD).

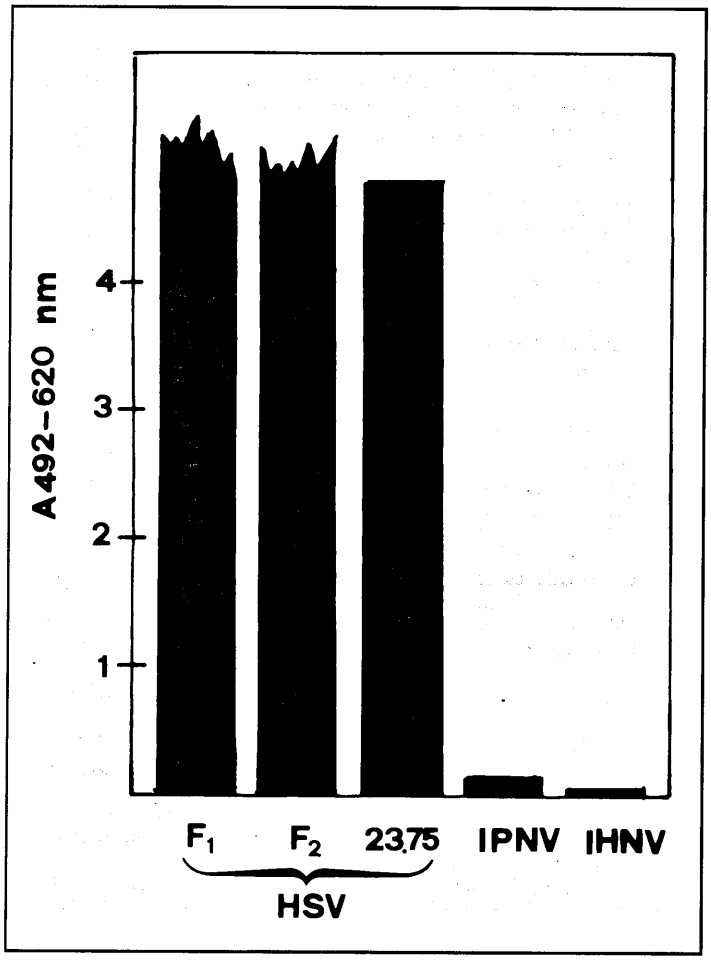

Figure 4—Specificity of HSV detection by the ELISA. Rhabdoviruses were purified as described and added at concentration of 50 ng/well. Infectious pancreatic necrosis virus (IPNV) was used as supernatant and was added at concentration of 50 µg/well. IHNV = infectious hematopoietic necrosis virus; F_1, F_2, and 23.75 are HSV strains.

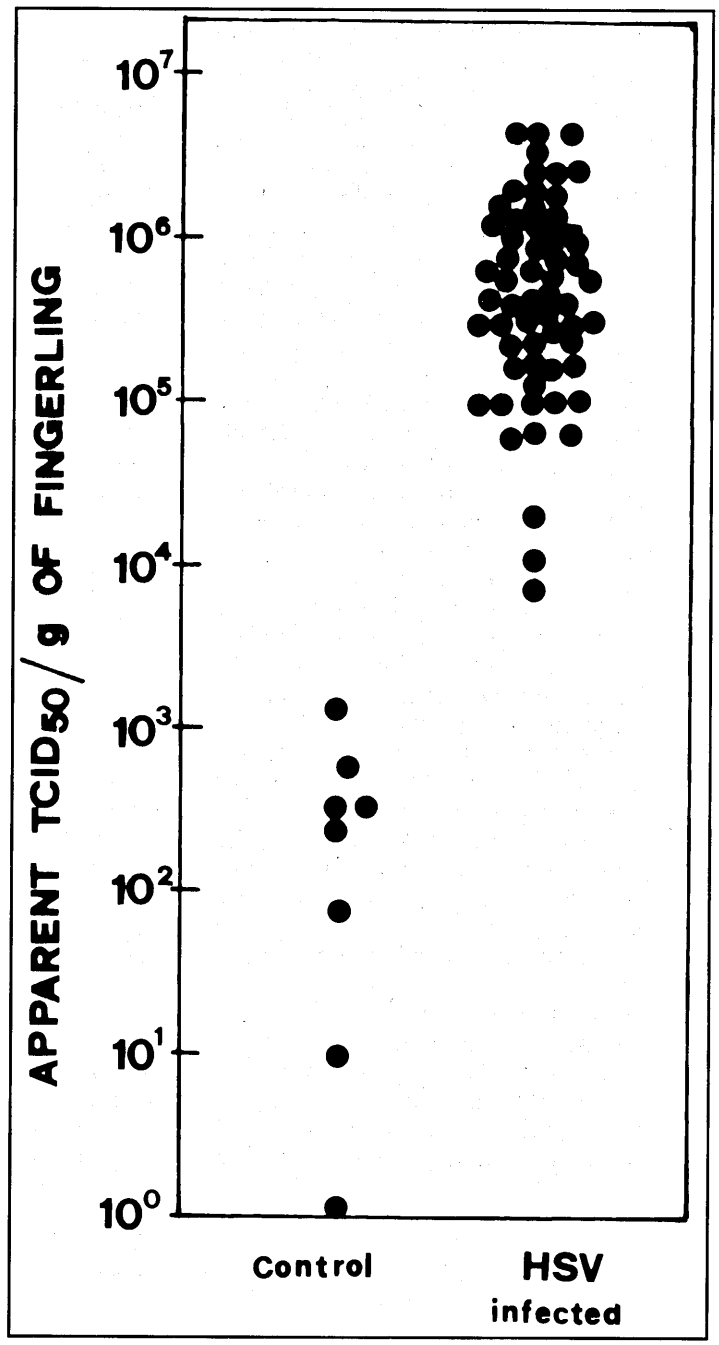

Figure 5—Apparent HSV concentration in samples from control and dead HSV-infected fingerling trout.

tested by ELISA simultaneously. Apparent $TCID_{50}$ per well was estimated by ELISA by comparison with a standard HSV supernatant titrated in EPC. Results were expressed in apparent $TCID_{50}$ per gram of trout (Fig 5). All fingerlings that died during the infection had > 7,000 apparent $TCID_{50}$/g. Apparent titer ranged between 7,000 and 6 x 10^6 $TCID_{50}$/g. Samples could be frozen and thawed 6 consecutive times without any loss in ELISA absorbance values.

Discussion

Monoclonal antibodies against HSV nucleoprotein N were selected to optimize the ELISA, because the N protein is the least variable protein of the rhabdoviruses in general. Thus, this assay could detect the highest number of viral serotypes and isolates,[28] not only F_1.[16] The epitopes defined by the MAB choosen were highly conserved in the 3 defined HSV serotypes and 5 Spanish isolates. The N protein of the virus is a major component found in the complete virus and in infected cells,[3,4] and it is the first protein synthesized after 1 to 2 hours of infection.[13]

First attempts to produce ELISA for detection of HSV were unsuccessful, apparently because the titer of the antisera was too low.[11] Virus could be detected in cell culture before cytopathic effect, and assays were specific for IHN or HSV viruses; however, sensitivity was still a problem.[10] Characterization of the epitope specificity of 19 HSV MAB and recovery of activity after conjugation with peroxidase led to the selection of a MAB with high titer, high yield, easily scaling up method for conjugation and high stability. This MAB was used as the conjugate in the assay. Our first attempts to use 2 noncompetitive HSV MAB against protein N for sandwich ELISA met with failure because of low sensitivity (1,000 ng/well). Sensitivity was improved from 1,000 ng of HSV/well at low ionic strength to 20 ng of HSV/well at ≥ 1*M* NaCl. Most probably, this is attributable to nucleocapsid disruption at such high ionic strength. By using the 1-step assay, sensitivity could be further improved to 0.2 ng of HSV/well. Most probably, this could be attributable to induced conformational changes by the binding of MAB 2C9 to the virus. Taking into account that N/Nx proteins comprise about 40% of the total viral proteins, the real sensitivity of the ELISA described is in the order of 0.1 ng of N protein/well, which is near the limit of these assays.[27] The highest reported ELISA sensitivity was 4 ng (10^3 $TCID_{50}$) of HSV or IHN virus in cell culture by use of immunodot analysis.[9] Schultz et al[29] developed a similar assay for detection of 10^2 $TCID_{50}$ of IHN viruses/10 µl of sample by use of MAB. However these techniques, to the contrary of what happens in our ELISA, could not be used with homogenates because their high protein content clogged the nitrocellulose filter.[30] The cut-off value to discriminate negative from positive results could be evaluated graphically as 10^3 apparent $TCID_{50}$/g (Fig 5). The ELISA technique also detected HSV antigen in cell cultures that had been used for isolating virus from infected fish when cytopathic effect was not evident or when toxicity of the fish extract masked development of viral effects (data not shown).

One hundred microliters of homogenate is sufficient for the assay. This microsample volume requirement stemming from the assay sensitivity is especially desirable for testing individual fingerlings. In addition, such samples as cell culture supernatants, with low HSV content, might now be assayed with high precision. The application to this ELISA of the highly stable reagents previously developed and optimized, such as, a 2-year stable dilution buffer at room temperature,[18] addition of phenol red to allow visualization of the wells pipetted and continuous monitoring of pH, possibility of using microtitation plates divided in rows that adapt the number of assays to the daily variable number of samples, low background peroxidase-*o*-phenylenediamine buffer with added merthiolate for increasing stability,[26] and simple conjugation to peroxidase,[24] makes the new method easily scalable, highly reproducible, and available for diagnostic field testing.[19] Homogenizers made with 96-rod plates can be used to obtain individual homogenates.[31] In this case, the individual homogenates can be transferred, using a multichannel pipette to the ELISA solid phase. Time to examine 40 samples can thus be < 4 hours, including homogenization and ELISA. Because the ELISA yielded a strong visual positive reaction with antigen from infected fish without any false-negative results (Fig 5), it might be possible to use it in its present form for diagnosis of the disease in fish in the field. Samples (entire fish) can be frozen and kept at −20 C before doing the ELISA.

However, the ELISA might not be as sensitive as cell culture for detecting carrier fish, and therefore, its use in routine screening of fish stocks is not yet recommended. Detection of HSV by ELISA should depend on the stage of infection (acute/carrier) and on the onset of host antibody formation. Virus titer could be found below the sensitivity margin in pooled samples, and therefore, it would be advisable to examine individual, rather than pooled, organ samples.

The binding to solid-phase HSV in the presence of high salt and detection of the virus, provide a nonprecipitation, nonagglutination, and nonradioactive alternative for assessment of HSV, with the advantages of stable reagents, easy automation, wide linear range, high reproducibility, low background, higher sensitivity than other immunoassay techniques, high precision and low prevalence of false-positive or -negative results. All these characteristics make this assay suitable for specific HSV detection. Detection of other rhabdoviruses by enzyme immunoassays based on antinucleocapsid antibodies might be improved by use of the principles described herein.[32,33]

References

1. De Kinkelin P. Le Virus d'Egtved. II Purification. *Ann Rech Vet* 1972;3:199–208.
2. Jimenez de la Fuente J, Marcotegui MA, San Juan ML, et al. Diagnosis of viral disease in salmonid farms in Spain. *Bull Eur Assoc Fish Pathol* 1988;271:3–4.
3. Basurco B, Coll JM. Spanish isolates and reference strains of viral haemorrhagic septicaemia virus show similar protein size patterns. *Bull Eur Assoc Fish Pathol* 1989;9:92–95.
4. Basurco B, Coll JM. Variabilidad del virus de la septicemia hemorrágica viral de la trucha en España. *Méd Vet* 1989;6:425–430.
5. Gilmore RD, Leong JC. The nucleocapsid gene of infectious hematopoietic necrosis virus, a fish rhabdovirus. *Virology* 1988;167:644–648.
6. Dietzschold B, Lafon M, Wang H, et al. Localization and immunological characterization of antigenic domains of the rabies virus internal N and NS proteins. *Virus Res* 1987;8:103–125.
7. Bernard J, Lecocq-Xhonneux F, Rossius M, et al. Cloning and sequencing the messenger RNA of the N gene of viral haemorrhagic septicaemia virus. *J Gen Virol* 1990;71:1669–1674.
8. Zimmer K, Wiegand D, Manz D, et al. Evaluation of five different methods for routine diagnosis of rabies. *J Vet Med* 1990;37:392–400.
9. McAllister PE, Schill WR. Immunoblot assay: a rapid and sensitive method for identification of salmonid fish viruses. *J Wildl Dis* 1986;22:468–474.
10. Way K, Dixon PF. Rapid detection of VHS and IHN viruses by the enzyme-linked immunosorbent assay (ELISA). *J Appl Ichthyol* 1988;4:182–189.
11. Dixon PF, Hill BJ. Rapid detection of fish rhabdoviruses by the enzyme-linked immunosorbent assay (ELISA). *Aquaculture* 1984;42:1–12.
12. Winton JR, Arakawa CK, Lannan CN, et al. Neutralizing monoclonal antibodies recognize antigenic variants among isolates of infec-

tious hematopoietic necrosis virus. *Dis Aquat Organism* 1988;4:199–204.

13. Lorenzen N, Olesen NJ, Jorgensen PEV. Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. *Dis Aquat Organism* 1988;4:35–42.

14. Flamand A, Wiktor TJ, Koprowsky H. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. I. The nucleocapsid protein. *J Gen Virol* 1980;48:97–104.

15. Ristow SS, Arnzen JM. Development of monoclonal antibodies that recognize a type-2 specific and a common epitope on the nucleoprotein of infectious hematopoietic necrosis virus. *J Aquat Anim Health* 1989;1:119–125.

16. Mourton C, Bearzotti M, Piechaczyk M, et al. Antigen-capture ELISA for viral haemorrhagic septicaemia virus serotype I. *J Virol Methods* 1990;29:325–334.

17. Martinez J, Coll JM. Preliminary clinical studies of C-reactive protein quantified by enzyme-linked immunoassay. *Clin Chem* 1987;33:2185–2190.

18. Martinez J, Coll JM. Selection and clinical performance of monoclonal anti C-reactive protein in ELISA quantitative assay. *Clin Chim Acta* 1988;176:123–132.

19. Coll JM. Reproducible solid-phase enzyme immunoassays. *Immunologia* 1991;10:73–85.

20. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

21. Sanchez C, Dominguez J, Coll JM. Immunoglobulin heterogeneity in the trout *Salmo gairdneri* (Richardson). *J Fish Dis* 1989;12:459–465.

22. Rueda AZ, Coll JM. Cloning of myelomas and hybridomas in fibrin clots. *J Immunol Methods* 1988;114:213–217.

23. Coll JM. Injection of physiological saline facilitates recovery of ascitic fluids for monoclonal antibody production. *J Immunol Methods* 1987;104:219–222.

24. Coll JM. Heme increases peroxidase-antibody activity in aged conjugates. *J Immunol Methods* 1987;104:259–263.

25. Coll JM. Development of a fast solid-phase enzyme immunoassay for C-reactive protein. *Rev Esp Fisiol* 1988;44:359–368.

26. Coll JM. Addition of reducing agents to the peroxidase-o-phenylenediamine buffer reduces background of enzyme-immunoassays. *Rev Esp Fisiol* 1989;45:41–46.

27. Tijssen P, Burdon RH, Knippenberg PH. Laboratory techniques in biochemistry and molecular biology. In: Burdon RH, Knippenberg PH, eds. *Practice and theory of enzyme immunoassay.* Amsterdam: Elsevier Science, 1985;15.

28. Schneider LG, Dietzschold B, Dierks RE, et al. Rabies group specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. *J Virol* 1973;11:748–755.

29. Schultz CL, McAllister PE, Schill WB, et al. Detection of infectious hematopoietic necrosis virus in cell culture fluid using immunoblot assay and biotinylated monoclonal antibody. *Dis Aquat Organisms* 1989;7:31–37.

30. Hsu YL, Leong JV. A comparison of detection methods for infectious hematopoietic necrosis virus. *J Fish Dis* 1985;8:1–12.

31. French-Constant RH, Devonshire AL. A multiple homogenizer for the rapid preparation of samples for immunoassays and electrophoresis. *Biochem Genet* 1987;25:47–48.

32. Bourhy H, Rollin PE, Vicent J, et al. Comparative field evaluation of the fluorescent-antibody test, virus isolation from tissue culture, and enzyme immunodiagnosis for rapid laboratory diagnosis of rabies. *J Clin Microbiol* 1989;27:519–523.

33. Ferris NP, Donalson AI. An enzyme-linked immunosorbent assay for the detection of vesicular stomatitis virus antigen. *Vet Microbiol* 1988;18:243–258.