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Highly Sensitive Immunoassay for Direct Diagnosis of Viral Hemorrhagic Septicemia Which Uses Antinucleocapsid Monoclonal Antibodies

C. MOURTON,[1]* B. ROMESTAND,[2] P. DE KINKELIN,[3] J. JEFFROY,[4] R. LE GOUVELLO,[5] AND B. PAU[1]

Unité de Recherche en Immunologie, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 9921, Faculté de Pharmacie, 34060 Montpellier,[1] Laboratoire de Parasitologie et Immunologie, Université de Montpellier III, 34000 Montpellier,[2] Unité de Virologie des Poissons, Institut National de la Recherche Agronomique, 78350 Jouy en Josas,[3] Centre National d'Etudes Veterinaires et Alimentaires, Laboratoire de Pathologie des Animaux Aquatiques, 29280 Plouzané,[4] and Sanofi Santé Nutrition Animale, 33501 Libourne,[5] France

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An antigen capture enzyme-linked immunosorbent assay (ELISA) based on the detection of the viral nucleocapsid (anti-N system) was developed for the diagnosis of viral hemorrhagic septicemia. Four monoclonal antibodies directed against the viral nucleocapsid were produced; they all recognized the four viral hemorrhagic septicemia virus (VHSV) serotypes. Three of these monoclonal antibodies were used in a new antigen capture ELISA. The efficiency of the anti-N system in detecting purified and crude viruses as well as the virus in infected-organ extracts and infected blood was compared with that of a recently described antigen capture ELISA based on the detection of viral envelope glycoprotein Gp (anti-G system). For the detection of purified virus, the anti-N system was found to be as sensitive as the anti-G system (detection limit, 1 ng of total viral protein per ml), but the anti-N system was much more sensitive than the anti-G system for the detection of crude VHSV I (detection limits, 1×10^4 PFU/ml versus 5×10^5 PFU/ml). In organ extracts, VHSV I could be detected by both systems 3 days postinfection. The signal for the assay of VHSV I in blood 24 h postinfection was higher with the anti-N system than the anti-G system. Furthermore, VHSV I could be detected in 80% of the brain samples of surviving trout by the anti-N system and also by the anti-G system, but with a lower signal. In conclusion, we have developed a highly sensitive immunoassay for VHSV I that is more rapid and easier to perform than the currently used plaque assay.

The viral hemorrhagic septicemia (VHS) virus (VHSV) infects rainbow trout (*Oncorhynchus mykiss*) (11) and several salmonid species. VHSV is responsible for significant losses under trout-farming conditions in continental Europe, but recently several isolates of VHSV were recovered from asymptomatic salmonid fish carriers in the northwest United States (2). Because clinical signs exhibited by fish with overt VHS are not pathognomonic and closely resemble those generated by another fish rhabdovirus, infectious hematopoietic necrosis virus, there is a great need to develop a specific diagnostic procedure, especially within the context of fish health surveillance programs.

Presently, the diagnosis of VHS is based mainly on virus isolation followed by virus identification by an immunological method, such as immunofluorescence or immunoperoxidase staining or an enzyme-linked immunosorbent assay (ELISA) (6, 8, 9); this procedure takes about 10 days to perform. Recently, we described an antigen capture ELISA (anti-G system) based on the detection of viral envelope glycoprotein Gp (16). In this report, we describe another antigen capture ELISA (anti-N system), based on the detection of the viral nucleocapsid by monoclonal antibodies (MAbs) that we recently produced and characterized. The detection of VHSV in cell culture supernatants, in organ extracts, and in whole blood of infected trout by the two antigen capture ELISA systems was compared.

MATERIALS AND METHODS

Virus production, isolation, and purification. All viruses were grown at 14°C, VHSV I (strain 07.71), VHSV II (strain He), VHSV III (strain 23.75), infectious hematopoietic necrosis virus (strain 32.87), spring-viremia-of-carp virus, eel rhabdovirus (strains C 30 and B 12), pike fry rhabdovirus, and hirame rhabdovirus (12) in *Epithelioma papulosum cyprini* (EPC) cells (10) and VHSV IV (strain 02.84), infectious pancreatic necrosis virus, and perch rhabdovirus in rainbow trout gonad cells (23). VHSV I, II, III, and IV were produced in large amounts and purified by methods described elsewhere (3, 5).

Preparation of the VHSV I nucleocapsid. The VHSV I nucleocapsid was purified by the method described for vesicular stomatitis virus (19). In brief, purified VHSV I was disrupted in 10 mM Tris-HCl (pH 8) containing 0.25 M NaCl and 1% Triton X-100 for 30 min at room temperature. The nucleocapsid particles were separated from virus proteins by centrifugation at 200,000 × *g* for 2 h at 4°C through a 50% (vol/vol) glycerol pad. The pellet was then resuspended in 10 mM Tris-HCl (pH 8).

Preparation of MAbs against the nucleocapsid. Four hybridomas were obtained from BALB/c mice immunized with

* Corresponding author.

purified nucleocapsids by the hybridization technique previously described (20). The anti-N MAbs were produced in ascites fluid and purified on a protein A–Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). Another hybridoma secreting an anti-N MAb (MAb F5) was previously obtained (1) from BALB/c mice immunized with purified VHSV I. MAb F5 was tested along with the four new MAbs.

Experimental infection and preparation of combined kidney and spleen extracts and blood samples. (i) Experimental infection. Rainbow trout (*O. mykiss*) weighing approximately 150 g were experimentally infected by immersion in aqueous suspensions of VHSV I (7×10^4 PFU/ml) for 3 h at 10 ± 1°C. The trout were then maintained in a flowthrough aquarium at 10 ± 1°C.

(ii) Preparation of blood samples. On days 1, 3, 5, 7, and 9, five infected trout and two uninfected trout were anesthetized by immersion in a 0.02% ethylene glycol monophenyl ether solution, and blood was collected in heparinized tubes. The tubes were kept in ice until the blood was assayed by the anti-G and anti-N systems described below.

Thirty days after experimental infection, 20 surviving trout were anesthetized, and blood was collected in glass tubes and allowed to clot; each serum sample was divided into two batches: one for the detection of VHSV I by both the anti-G and the anti-N systems and the other for the detection of anti-VHSV antibodies by the serum neutralization test described below. There was not enough serum to determine the virus titer. Organ extracts were also prepared from these 20 surviving trout (see below).

(iii) Preparation of organ extracts. Ten infected trout were sacrificed daily for 11 days by immersion in an ethylene glycol monophenyl ether solution. The kidneys and spleens were pooled into two groups of five and cut into small pieces. The two groups of fragments were then subdivided into two batches, I and V, and ground with an ice-cold mortar and pestle. The two homogenates of batch V (V1 and V2) were diluted 1/10 (wt/vol) in medium V (Stoker's medium [21] supplemented with 2% fetal calf serum and 10% tryptose phosphate and buffered with 160 mM Tris-HCl [pH 7.6]; this was the medium used for the virological assay of VHSV I), clarified by centrifugation at 2,000 × *g*, and used to determine the virus titer. The two homogenates of batch I (I1 and I2) were diluted 1/10 (wt/vol) in medium I (medium V containing 2% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride [PMSF; Sigma, St. Louis, Mo.]), clarified, and used to determine the antigen level by the anti-G and anti-N systems. The antigen level in batches V1 and V2 was also assayed by the anti-G and anti-N systems. The kidneys and spleens from five uninfected trout were pooled, homogenized, and diluted in medium I (batch C).

Thirty days after infection and after blood collection, the 20 surviving trout were sacrificed, and the kidneys, spleens, and brains were removed. The kidneys and spleens were pooled into groups of two, and the brains were pooled into groups of four. The preparations were ground with an ice-cold mortar and pestle; the homogenates were then diluted in either medium I or medium V. Homogenates I were assayed by the anti-G and anti-N systems to detect viral antigens, and homogenates V were assayed by the plaque assay to determine the virus titer.

ELISA techniques. (i) Indirect ELISA. The hybridomas secreting anti-N MAbs were selected by use of an indirect ELISA. In brief, microtiter plates (Immunoplate II; Nunc, Roskilde, Denmark) were coated with purified VHSV I at 5 μg/ml or purified nucleocapsid at 5 μg/ml in phosphate-buffered saline (PBS) (pH 7.2) overnight at 4°C. The plates were washed with PBS containing 0.1% Tween 20 (PBS-T) and then incubated with hybridoma cell culture supernatants or purified MAbs. After incubation for 2 h at 37°C and washing, anti-mouse immunoglobulin G (whole molecule)–horseradish peroxidase (HRPO) conjugate (Sigma) in PBS-T was added, and the plates were incubated for 1 h at 37°C. After washing, the enzyme activity was revealed by the addition of enzyme substrate (4-mg/ml solution of *o*-phenylenediamine [Sigma] in 0.1 M citrate buffer [pH 5.0] containing 0.03% hydrogen peroxide). After incubation for 20 min, the reaction was stopped by the addition of 50 μl of 4 N H_2SO_4; the A_{490} was then measured.

(ii) Direct ELISA. Each of the five anti-N MAbs was coupled to HRPO (Boehringer Mannheim, Meylan, France) to prepare HRPO-MAb conjugates (17). The activity of the anti-N MAbs conjugated to HRPO was measured by a direct ELISA. In brief, microtiter plates were coated with 5 μg of purified VHSV I per ml overnight at 4°C. After washing, an HRPO-MAb conjugate serially diluted in PBS-T was added. Following incubation for 2 h at 37°C, the microtiter plates were washed again, the HRPO substrate solution was added, and the A_{490} was determined. The reactivity of the HRPO-MAb conjugates was measured against the four serotypes of VHSV by the direct ELISA.

(iii) Competitive inhibition of the binding of enzyme-labeled MAbs. The spatial relationships of the antigenic determinants were studied by competitive inhibition of the binding of each HRPO-MAb to purified virus by the same MAb or another unlabeled competitor MAb. The concentration of HRPO-MAb was chosen to yield a final A_{490} of 1.0 in the absence of a competitor. Unlabeled MAb (50 μl) and HRPO-MAb (50 μl) were added simultaneously to the coated wells. The plates were incubated for 2 h at 37°C and washed, and then the enzyme substrate was added.

(iv) Determination of the K_a. The apparent equilibrium association constant (K_a) of each MAb was determined by monitoring the inhibition of the binding of the HRPO-MAb in the presence of increasing concentrations of the unlabeled MAb. The procedure was the same as that for the competitive inhibition assay. We verified that the HRPO-MAb and the unlabeled MAb had the same binding activities. The K_as were calculated by Scatchard analysis of the ELISA data. Since the total amount of active HRPO-MAb must be known to calculate the K_a, we determined it by a serial binding experiment. One hundred microliters of HRPO-MAb diluted in PBS-T was added to a well coated with purified virus. After incubation for 2 h, the supernatant was transferred to a second virus-coated well. This procedure was continued until the absorbance value reached the background level (about five transfers). The sum of the absorbances of the different wells represented the total amount of active HRPO-MAb.

Antigen capture ELISA: anti-G system and anti-N system. (i) Detection of purified and crude viruses. When the anti-G MAbs were used, the antigen capture ELISA was performed as previously described (16). In brief, the microtiter plates were coated with an anti-G MAb (MAb A17) in PBS at 1 μg per well and incubated overnight at 4°C. They were then washed with PBS-T. The antigen (purified or crude virus) and the HRPO-MAb L7 conjugate were added simultaneously (100 μl of each), and the plates were incubated for 3 h at 37°C. The enzyme activity was revealed after the addition of the substrate.

When the anti-N MAbs were used, the solid phase antibody was a mixture of MAb 1E5 and MAb 5F10 (5 μg of each per ml) in PBS, and the labeled MAb was a mixture of

HRPO-MAb 9A9 and HRPO-MAb 5F10 conjugates (50 ng of each conjugate per ml). The assay was performed as described above for the anti-G system.

The limit of detection was defined as the protein concentration (for purified virus) or the number of PFU per milliliter (for crude virus) giving a signal equal to the mean of 10 reagent blanks (purified virus was replaced by PBS-T and crude virus was replaced by EPC cell supernatant) plus 2 standard deviations.

(ii) Detection of virus in organ extracts, whole blood, and serum. One hundred microliters of homogenate prepared as described above or whole blood or serum from infected or uninfected trout and 100 μl of anti-G MAb–HRPO (L7) or anti-N MAb–HRPO (5F10 and 9A9) conjugate were added simultaneously to wells coated with anti-G MAb A17 or anti-N MAbs 5F10 and 1E5. The plates were incubated overnight at 4°C; the assay was then performed as described above.

Detection of anti-VHSV antibodies by the serum neutralization test. VHSV-neutralizing activity in sera from infected or uninfected trout was determined by a plaque assay (7). In brief, the sera were heated for 30 min at 45°C to inactivate complement. Serial twofold dilutions of the sera were then mixed with a constant amount of VHSV I (500 PFU per well), and the mixture was incubated overnight in the presence of trout complement. EPC cells (2×10^5) were then added to the serum-virus mixture, and the plates were incubated for 3 days at 14°C. The cells were fixed with a 10% formalin solution and then stained with a 0.1% crystal violet solution. The titer is expressed as the highest dilution yielding significant protection of the cell monolayer, compared with the destruction of cells in the presence of negative control sera.

Plaque assay for the determination of the virus titer. A homogenate (batch V) serially diluted in medium V supplemented with 50 μg of DEAE-dextran per ml was added to a 24-h EPC cell monolayer. After adsorption for 1 h at 14°C, 2 ml of twofold-concentrated medium V supplemented with 0.3% agarose was added. After incubation for 3 days, the cells were fixed and stained as described above. The plaques were counted, and the PFU were calculated.

RESULTS

Production and characterization of anti-N MAbs. Four hybridomas secreting anti-N MAbs (designated 1E5, 5F10, 9A9, and 8B8) were selected by an indirect ELISA with purified virus or purified nucleocapsids. The MAbs were all immunoglobulin G1. Their K_as, calculated by Scatchard analysis, ranged from 1.3×10^8 to 2.0×10^{10} M^{-1}. Anti-N MAb F5 was of the immunoglobulin G2b isotype; its K_a was 8×10^9 M^{-1}. When the direct ELISA was used to determine the specificity of the HRPO–anti-N MAbs, all four serotypes were recognized to the same extent by the five MAbs.

Using the competitive inhibition test, we determined that three different antigenic domains (I, II, and III) were defined on VHSV I by the anti-N MAbs (Fig. 1). Domains I and II were partially overlapping.

Antigen capture ELISA: anti-N system and anti-G system. To detect the nucleocapsid, we developed an antigen capture ELISA involving three different MAbs: MAbs 1E5 and 5F10 as capture MAbs and a cocktail of HRPO-MAb 5F10 and HRPO-MAb 9A9 as tracer MAbs. This combination proved to be the most efficient of all those we tested (data not shown). We compared the efficiencies of the anti-N system and the anti-G system for the detection of purified and crude viruses. The anti-N system was as sensitive as the anti-G system for purified virus; we were able to detect as little as 1 ng of total viral protein per ml in a purified virus preparation by using the two systems. For crude virus, the anti-N system was more sensitive; the detection limits were 1×10^4 PFU/ml in the anti-N system and 5×10^5 PFU/ml in the anti-G system (Fig. 2). The anti-N system was specific for the four serotypes of VHSV; none of the other viruses was recognized by these MAbs.

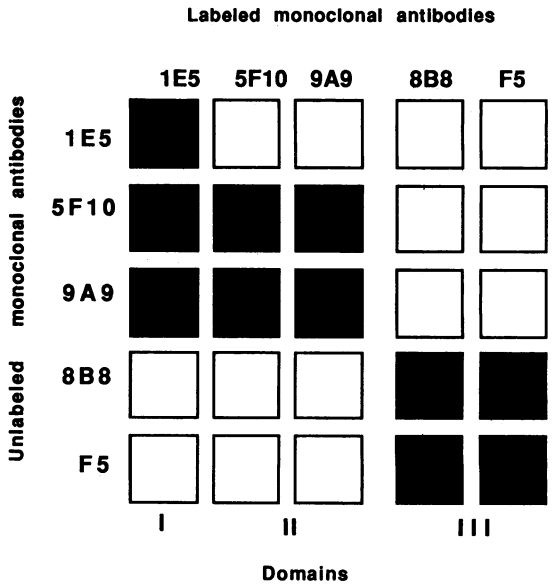

FIG. 1. Schematic representation of the competitive inhibition of the five HRPO–anti-N MAbs by the five unlabeled MAbs. Symbols: ■, complete inhibition (>80%); □, absence of inhibition (<80%).

Diagnosis of trout infection by the antigen capture ELISA systems. (i) Detection of virus in organ extracts. The detection of VHSV I in organ extracts from experimentally infected trout by the anti-G and anti-N systems was improved in the presence of Triton X-100 and PMSF (Fig. 3). The virus was detected by both immunoassays (A_{490}, >3) 3 days postinfection in the two batches I1 and I2. On days 10 and 11, the samples still showed an A_{490} of >3 in the anti-N system, whereas the signal in the anti-G system was lower. Thirty days postinfection, the virus could be detected in only 4 kidney and spleen extracts of 10 by both the anti-N antigen capture ELISA and the plaque assay (data not shown). The virus was detected in 80% of the brain samples from trout 30 days postinfection by the anti-N and anti-G systems, but the signal was somewhat lower in the latter immunoassay (Fig. 4).

(ii) Detection of virus in blood. The detection of VHSV I in blood from experimentally infected trout by the anti-G and anti-N systems was compared. The virus was detected from 24 h up to 7 days postinfection mainly by the anti-N system (Fig. 5). Thirty days postinfection, the virus could not be detected in any of the blood samples from the surviving trout by the immunoassays (data not shown). The virus titer in blood was not determined, however, because of the limited amounts of samples available.

Diagnosis of VHSV I in organ extracts by the plaque assay. We determined the virus titer in homogenates diluted in medium V by the plaque assay. By this method, the virus was detectable on days 1 and 2 but only in one of the two samples tested; the titer was 10^3 PFU/ml (Fig. 6). Such a

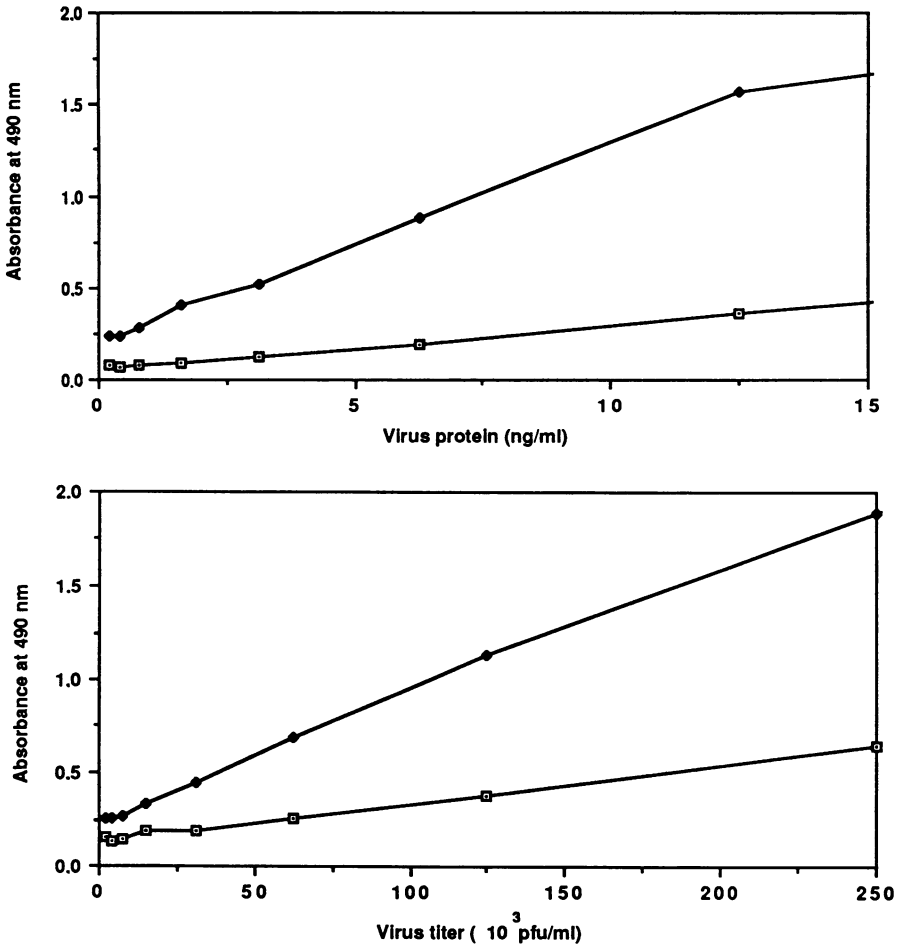

FIG. 2. Standard curves for the two antigen capture ELISA procedures. Symbols: ⊡, anti-G system; ◆, anti-N system.

small number of PFU per milliliter could not have been detected by the antigen capture ELISA systems because their limit of detection is higher. Thirty days postinfection, the virus was detected in 3 of the 10 extracts; 2 were the same samples of spleen and kidney found to be positive by the anti-N system (data not shown). The virus was detected in all brain samples 30 days postinfection (Fig. 7).

Detection of antibodies in trout. By the serum neutralization test, antibodies were found in sera from 15 of 20 surviving trout 30 days postinfection. The antibody titer, defined as the reciprocal of the dilution yielding about 50% neutralization, ranged from 80 to 2,560 (data not shown).

DISCUSSION

In the present paper, the immunological assay that we previously described (16) for the detection of VHSV glycoprotein G is compared with a new immunological assay designed to detect the VHSV nucleocapsid.

First, we tried to improve the assay of VHSV I in organ extracts of experimentally infected trout by the anti-G system. The results show that medium I enhanced the immunological detection of VHSV I. The presence of Triton X-100 in medium I probably rendered epitopes on the glycoprotein more accessible to the MAbs. However, since the limit of detection of the anti-G system was still not as low as that of the plaque assay and since the anti-G system only detects VHSV I and VHSV II, we decided to produce and characterize anti-N MAbs. Cell fusion performed following immunization of mice with VHSV I nucleocapsids generated four hybridomas secreting MAbs reacting specifically with representative strains of the VHSV group in the indirect ELISA. Two other anti-N MAbs have been described, namely, MAb F5 (1) and MAb IP5B11 (14); they were obtained by immunizing mice with purified virus.

The four anti-N MAbs described in the present report defined three antigenic domains on the nucleocapsid: domain I, defined by MAb 1E5, and domain II, defined by MAbs 9A9 and 5F10, were partially overlapping; a third, independent one, domain III, was defined by MAbs 8B8 and F5. MAb IP5B11 was not tested.

The MAbs used in the antigen capture ELISA were selected on the basis of their capacity to capture VHSV I and their ability to detect the other representative strains of the VHSV group. All five MAbs were tested as capture antibodies and as labeled second antibodies. We found that the most sensitive system for VHSV involved MAbs 5F10 and 1E5 as capture antibodies and MAbs 5F10 and 9A9 as tracer antibodies.

Compared with the previously described anti-G antigen capture ELISA system (16), the present anti-N ELISA showed the same sensitivity for purified virus but a higher sensitivity for crude virus: i.e., 1×10^4 PFU/ml versus 5×10^5 PFU/ml. This result was probably due to the larger

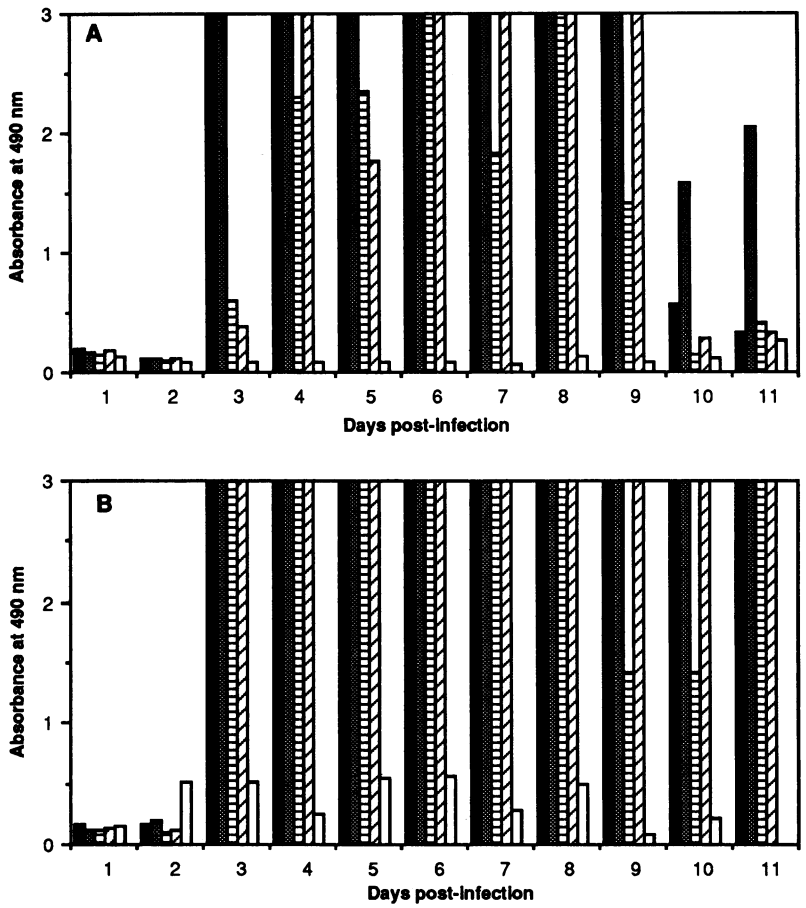

FIG. 3. Detection of VHSV I antigens in organ extracts by the anti-G (A) and anti-N (B) antigen capture ELISA systems. Symbols: ■, batch I1; ▩, batch I2; ▤, batch V1; ▨, batch V2; □, batch C. Batches I1 and I2 correspond to two samples of the kidney and spleen homogenate prepared from five infected trout and diluted in medium I (containing 2% Triton X-100 and 2 mM PMSF). Batches V1 and V2 correspond to two samples of the kidney and spleen homogenate prepared from five infected trout and diluted in medium V. Batch C corresponds to a kidney and spleen homogenate prepared from three uninfected trout and diluted in medium I.

number of virus subunits released in the crude virus preparation than in the purified virus material. Indeed, many nucleocapsids can be lost during the course of purification because their density is higher than that of the whole virus.

The anti-N system was found to be specific for all four representative strains of the VHSV group, whereas the anti-G system only recognized VHSV I and VHSV II.

The use of the anti-N antigen capture ELISA system for

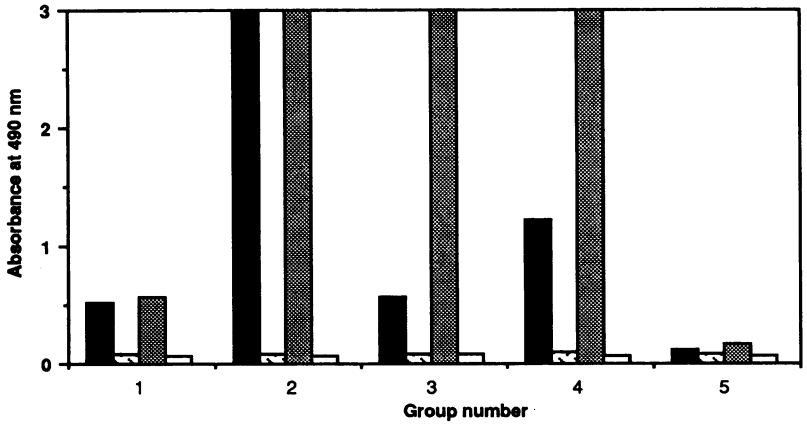

FIG. 4. Detection of VHSV I in the brains of surviving trout by the anti-G (■) (control: ▩) and anti-N (▭) (control: □) systems. Brain homogenates were prepared from four infected trout and diluted in medium I. Control brain homogenates were prepared from four uninfected trout and diluted in medium I.

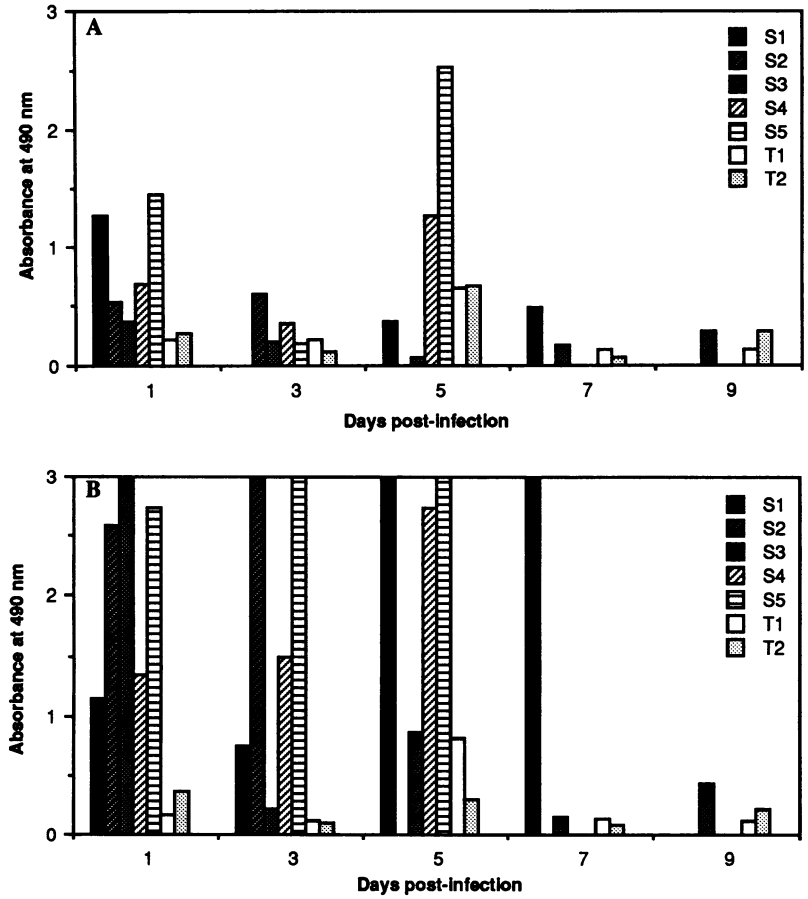

FIG. 5. Detection of VHSV I in blood by the anti-G (A) and anti-N (B) systems. S1, S2, S3, S4, and S5: blood samples from five infected trout; T1 and T2: blood samples from two uninfected trout. Blood was collected in heparinized tubes; the tubes were maintained in ice until the blood was assayed. Trout corresponding to S2 died on day 4, those corresponding to S4 and S5 died on day 6, and those corresponding to S1 died on day 8. The trout corresponding to 53 was still alive on day 9.

the diagnosis of VHS during the course of an experimental infection in rainbow trout deserves several comments. When applied to VHSV screening of extracts of pooled kidneys and spleens, the fish organs most often used for viral detection, the anti-N and anti-G systems detected VHSV on day 3 postinfection. It took one more day to detect the virus with these antigen capture systems than with the plaque assay; however, since the plaque assay requires 3 days to

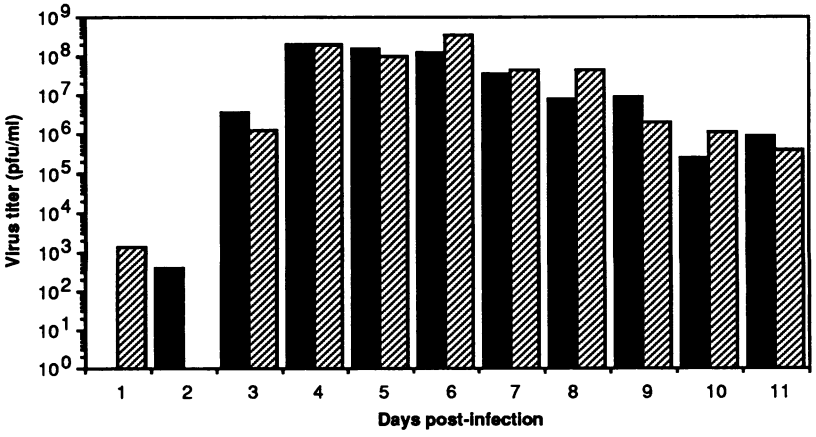

FIG. 6. Virus titer in organ extracts. Symbols: ■, batch V1; ▨, batch V2. Each bar represents the number of PFU in an extract of a pool of two kidneys and spleens obtained from infected trout and diluted in medium V.

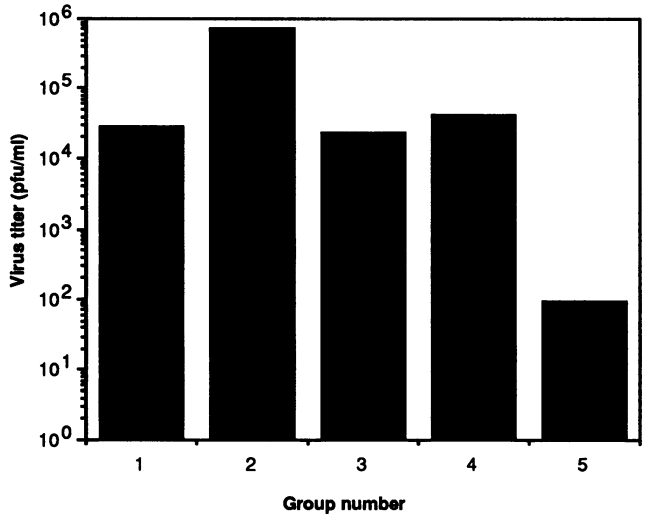

FIG. 7. Virus titer in the brains of surviving trout. Each bar represents the number of PFU in a brain extract obtained from four trout and diluted in medium V.

perform, compared with only 1 day for the antigen capture systems, the results are obtained earlier by the immunoassays. On days 1 and 2, the virus was detected in one of the two samples by the plaque assay. After 3 days of infection, the two ELISA systems yielded identical positive responses for about 9 days; the virus titer was between 10^4 and 10^8 PFU/ml over this period.

Finally, at 1 month postinfection, the positive responses obtained by both the anti-G and the anti-N systems and by the plaque assay for the kidneys and spleens dropped to 20% of the samples tested. In contrast, most of the brain samples were positive when assayed on day 30 postinfection by the anti-N ELISA and by the anti-G ELISA, but with a low signal. The shift of VHSV localization to the brain as fish become asymptomatic virus carriers corroborates previous reports (4, 18). However, the high reactivity of the brain extracts in the anti-N ELISA, associated with a virus titer of about 10^4 PFU/ml, and a low signal in the anti-G ELISA suggest that viral antigens, namely, nucleocapsids, accumulate to high levels in certain central nervous system cells; this accumulation probably corresponds to the anti-VHSV immunoperoxidase-reacting cells described in recent histopathological studies of VHS infection (13).

Whereas the previously described ELISA procedures can only be used for the diagnosis of overt fish virus infections (6, 16, 22), the anti-N antigen capture ELISA described here is a suitable virus screening method for the detection of asymptomatic virus carriers. Access to the central nervous system for sampling can be greatly facilated by use of a cork bore as described for the diagnosis of the trout parasite *Myxosoma cerebralis* (15).

An interesting finding obtained with the use of the anti-N ELISA system was the positive response recorded with blood from trout 24 h postinfection. This result suggests early viral infection of some circulating leucocytes, in contrast to results described elsewhere (4, 18), which suggested that viremia could not be detected 24 h postinfection. With the anti-G ELISA system, the signal was either low or absent. Furthermore, VHSV could be detected in blood in all trout for up to 5 days postinfection by the anti-N ELISA system. The feasibility of using blood samples for the diagnosis of an overt VHS infection might simplify the sampling and further processing procedures required in fish health surveillance programs.

In conclusion, the ELISA systems presently used for the diagnosis of rhabdoviral infections are based on polyclonal antibodies. For VHS diagnosis, ELISA systems based on rabbit anti-VHSV serum lack sensitivity and specificity and show great variability in their reactivity (6, 22). For the detection of virus in infected organs with polyclonal antibodies, serum must be absorbed with uninfected organs. In addition, the sensitivity of these polyclonal antibody ELISA procedures in detecting virus in cell cultures is lower than that of our anti-N ELISA system (10^5 to 10^6 PFU/ml versus 10^4 PFU/ml). Therefore, our anti-N system seems to be suitable for the detection of clinical infections and healthy carriers and for the identification of virus in cell cultures. Presently, the anti-N system is under study as a means of diagnosing VHS in trout under hatchery conditions.

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