ONE-STEP ELISA TO DETECT WITH A UNIQUE PROTOCOL INFECTIOUS PANCREATIC NECROSIS AND VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUSES IN TURBOT HOMOGENATES

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SUMMARY

A unique protocol to select and use non-competitive monoclonal antibodies (MAbs) for two one-step sandwhich ELISAs has been developed to detect both infectious pancreatic necrosis (IPN) and viral haemorrhagic septicaemia (VHS) viruses mixed with turbot kidney homogenates. For maximal sensitivity, the turbot kidney homogenates seeded with the viruses could be diluted ≥ 40-fold with high-ionic strength buffers and assayed for the presence of VHS rhabdovirus nucleoproteins (N/Nx) or IPN birnavirus structural protein (VP3). Preliminarly attemps show that the test was able to detect both viruses in laboratory infections of a few young turbot.

KEY WORDS: ELISA
Monoclonal antibodies
Viruses
Turbot

INTRODUCTION

Current methods of fish virus identification are based upon cell culture and serum-neutralization. These tests are time-consuming, labour-intensive and require well prepared laboratories. Other serological techniques used for fish virus detection and identification, such as immunofluorescence or immunoblot, have limited application because of either low sensitivity, technical complexity, difficulty of interpretation or need for specialized equipment (McAllister, Schill, 1986; Way, Dixon, 1988). The enzyme linked immunosorbent assay (ELISA) technique using polyclonal antibodies (PAbs) has been adapted to detect the salmonid rhabdoviruses, causing infectious haematopoietic necrosis (IHN) (Dixon, Hill, 1984) and viral haemorrhagic septicaemia VHS (Way, Dixon, 1988) as well as infectious pancreatic necrosis (IPN) (Babin *et al.*, 1990, 1991; Domínguez *et al.*, 1990, 1991). Monoclonal antibodies (MAbs) have improved the sensitivity of both immunofluorescence and ELISA (Sanz, Coll, 1992a, b; Mourton *et al.*, 1990). A

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sensitivity of 0.2-1 ng/ml has been found for VHSV F_1 by using MAbs against the G glycoprotein (Mourton *et al.*, 1990) or for all VHSV serotypes by using MAbs against the nucleoproteins (N/N_x) (Sanz, Coll, 1990, 1992a; Mourton *et al.*, 1992), respectively. Advantages of one-step ELISA include rapid processing of large numbers of samples using simple equipment, low incidence of errors and short-time processing (Martinez, Coll, 1987, 1988; Coll, 1991).

The recent demonstration that aquaculture reared turbot (Scophthalmus maximus, L) could be a natural host for salmonid rhabdovirus has made rapid viral diagnosis important for this fish species. In 1984 Castric and De Kinkelin demonstrated the susceptibility of turbot and sea bass to viral haemorrhagic septicaemia virus (VHSV) by experimental infections and recently, Schlotfeldt *et al.*, (1991) reported a natural outbreak among farmed turbot *(Scophthalmus maximus, L).* Infectious pancreatic necrosis virus (IPNV) has also been isolated in Norway from a population of turbot fry exhibiting a 40 p. 100 mortality (Mortensen *et al.*, 1991 personal communication) and young turbot has been shown to be its host (Castric *et al.*, 1987). An ELISA method to detect all the possible viruses affecting turbot would be beneficial for aquaculture.

This study describes an immunoenzymatic method with a unique one-step protocol that detects proteins of both IPN and VHS viruses in turbot kidney homogenates after its seeding with the cell culture-grown viruses or in laboratory infections. The test uses two non-competitive MAbs described before in a sandwich ELISA. This method must now evaluated against direct isolation procedures to establish the potential of its use during turbot viral disease outbreaks.

MATERIAL AND METHODS

Viruses

The viruses used were VHS-F_1 provided by Dr. P. E. Vestergaard-Jorgensen (NVRL, Denmark) and IPN-Sp provided by Dr. Babín (INIA in Madrid). Other isolates used have been described by Domínguez *et al.*, (1991) and Sanz, Coll (1992).

Epithelioma papullosum cyprini (EPC) cells were used throughout all experiments. Cell culture techniques (RPMI-1640 medium, Flow, Ayrshyre, Scotland), calculation of virus titers as tissue culture infectious doses ($TCID_{50}$) and virus purification were performed as described by Basurco, Coll (1989a). The protein content of purified virus was calculated by separating the viral proteins by SDS gel electrophoresis (Basurco, Coll, 1989b), staining with Coomassie blue and comparing to standards. Viral purity as calculated from the scan of Coomassie-blue stained electrophoresed proteins was about 95 p. 100.

Monoclonal antibodies (MAbs)

The production and characterization of MAbs against VHSV and IPNV has been described (Sanz, Coll, 1992a; Babin *et al.*, 1990; Domínguez *et al.*, 1991). A description of the characteristics of the MAbs selected for this work is given in Table 1.

TABLE 1

CHARACTERISTICS OF MAbs SELECTED FOR SANDWICH ELISA

Características de los anticuerpos monoclonales seleccionados para el ELISA sandwich

Virus	Use	Clone	Antigen	Isotipe	Specificity
VHSV	Solid-phase	2D5	VHS-144	G_1, K	N/Nx
	Conjugate	2C9	VHS-144	G_{2a}, K	N/Nx
IPNV	Solid-phase	2A1	IPN-Sp	G_{2a}, K	VP_3
	Conjugate	2F12	IPN-Sp	G_{2a}, K	VP_3

All antibody pairs choosen were specific for a single virus and the sandwhich assay was negative for the other virus. Each of the pairs of MAbs were directed against different antigenic determinants on the same viral proteins (N/Nx for VHSV and VP_3 for IPNV) as defined by competition experiments. The generation and characterization of the MAbs anti-IPN, sA1 (Babin *et al.*, 1990) and 2F12 (Dominguez *et al.*, 1991) have already been described and recognized two epitopes shared by all the representative strains of the 9 IPNV serotypes proposed by Hill, Way (1983) (Babin *et al.*, 1991). The generation and characterization of the MAbs anti-VHSV, 2D5 and 2C9 (Basurco *et al.*, 1991; Sanz, Coll, 1992a, b) have already been described and recognized two epitopes shared by all 3 international reference serotypes and 5 Spanish isolates (Sanz *et al.*, 193)

Todas las parejas de anticuerpos escogidas fueron específicas para cada virus y el ensayo sandwich fue negativo para el otro virus. Cada una de las parejas de anticuerpos monoclonales estaban dirigidas contra diferentes determinantes antigénicos en las mismas proteínas víricas (N/Nx para VSHV y VP3 para VNPI) según el resultado de los experimentos de competición. La generación y caracterización de los AcM anti-NPI 2A1 (Babin et al., *1990) y 2F12 (Domínguez* et al., *1991) según se han descrito con anterioridad reconocen epítopos comunes en los 9 serotipos de NPI propuestos por Hill, Way (1983) (Babin* et al., *1991). La generación y caracterización de los AcM anti VDHV, 2D5 y SC9 (Basurco* et al., *1991; Sanz, Coll, 1992a, b) según se ha descrito con anterioridad reconocen epítopos comunes en los 3 serotipos de referencia internacional y en 5 aislados españoles (Sanz* et al., *1993)*

Obtention of ascites and purification of the MAbs

Ascites was obtained from hybridoma-injected mice as described by Coll (1987a). The ascites was clarified by low speed centrifugation and stored at –40 °C. The ascites was purified by affinity chromatography over protein A-Sepharose columns (Pharmacia, Upsala, Sweden). Sample buffer was 1.5 M glycine, 3 M NaCl, pH 8.9. The retained IgG was eluted with 0.1 M citric acid pH 4.9. Eluted fractions were pooled and dialized against 10 mM sodium phosphate, 150 mM NaCl, pH 7.2 (PBS). Purity tested by polyacrylamide gel electrophoresis (Basurco, Coll, 1989b) gave two single Coomassie blue stained bands at 50 and 24 KDa, respectively.

Indirect ELISA

Ascites, purified MAbs and conjugates were titrated by an indirect ELISA. Briefly, 1 µg of virus was added to the wells of polystyrene plates in 100 µl of distilled water, dried overnight at 37 °C, washed with washing buffer (described here), dried and kept at 4 °C with dessicant. About a 5-fold increase in absorbance was obtained by coating by drying the plates instead of by coating using the carbonate/bicarbonate based buffers (Coll, 1991; Sanz, Coll, 1992a). The MAbs were diluted in dilution buffer (described here) and 100 µl added to the wells. After 1 h

incubation and washing, 100 µl of peroxidase-labelled anti-mouse IgG (Nordic, The Netherlands) or MAb, were added and incuabated for 30 min. Other details as described later.

Peroxidase conjugates

To couple peroxidase to MAbs 1 mg of purified and dialysed anti-virus antibodies were mixed with 25 mg of horseradish peroxidase (E.C. 1.11.1.7., 1000 U/mg, RZ/3.3, Boehringer Mannheim), in 1 ml of PBS pH 7.2 and 20 µl of 25 p. 100 aged-glutaraldehyde. After incubation at 37 °C for 30 min, 1 ml of 4 M glycine in PBS was added and the mixtures were incubated overnight at room temperatura. The resulting conjugates had a molar ratio of antibody to peroxidase of 0.9 to 1 (Coll, 1987b). The conditions for couplin of each of the MAbs were optimized individually. Recovery of activity after conjugation ranged from 41 to 80 p. 100 depending on the MAb as an alyzed by indirect ELISA.

One-step sandwich enzyme immunoassay

The one-step sandwich enzyme immunoassay method was developed for capture and detection of virus by using procedures developed before (Coll, 1988, 1989). The solid-phase was Nunc microtiter polystyrene plates divided in rows of 8 × 2 wells (Microwell module F-16, medium binding capacity) which easily adapt to the number of samples. Polystyrene plates were coated with 1 µg/well of anti-virus MAb (Table 1) in 100 µl of distilled water, dried overnight at 37 °C and blocked by washing with 10-fold diluted dilution buffer. Plates were dried 2 h at 37 °C and kept sealed with dessicant at 4 °C. Negative control plates coated with mouse IgG_1 or IgG_{2a} (Chemicon Int. Temecula, CA) were used in parallel ELISA. Coated, blocked and dried solid-phases were stable for at least 1 month at 4 °C.

To assay, 50 µl of supernatant fluid from turbot kidney homogenates (10 p. 100 w/v in cell culture medium with 2 p. 100 fetal calf serum and 20 mM Hepes) was combined with 50 µl of 2x concentrated dilution buffer (final concentration, 2 mM KCl, 8 mM Na_2HPO_4 1.4 mM KH_2PO_4, 1 M NaCl, 0.24 mM merthiolate or thimerosal, 5 g/l bovine serum albumin, 4 g/l Tween 20, 50 mg/l phenol red, pH 7.8) containing a 125-fold dilution of the conjugate. Plates were incubated for 120 min at room temperature and washed 4-times with ten-fold diluted dilution buffer. Then, 100 µl of substrate buffer (150 mM sodium citrate, 3 mM H_2O_2, 0.24 mM merthiolate and freshly added 1 mg/l of o-phenylenediamine, pH 4.8) was added and the reaction stopped with 50 µl of 4 M H_2SO_4 after 30 min. The plates were read in a Titertek Multiskan at three wavelengths (450, 492 and 620 nm). The absorbance at 620 nm was used to correct for individual variation between wells. The absorbance at 450 nm was used to calculate the 492 nm absorbance values ≥ 2 by aproximation (absorbance at 492 nm = absorbance at 450 nm × 2.4). Sensitivity was calculated with a 95 p. 100 confidence level by assuming a normal distribution of purified virus background values as suggested by Tijssen (1985).

Addition of viruses to turbot kidney extracts

After health inspection, healthy turbot *(Scophthalmus maximus, L)* (2-3 kg per fish) were purchased from commercial farms. Kidneys from turbot were excised,

pooled, and homogeneized with a Potter-Elvehjem homogenizer (a glass tube provided with a pestle) at 10 p. 100 w/v in cell culture medium. After centrifugation at 3000 g for 20 min, the supernatants were diluted 1: 1 with cell culture supernatants from EPC infected monolayers with either VHSV or IPNV (Basurco, Coll, 1989a, b). Final virus concentration in the mixtures (20-fold diluted) was estimated by the $TCID_{50}$ and contained 5×10^6 $TCID_{50}$/ml of VHSV and 5×10^7 $TCID_{50}$/ml of IPNV. Control extracts were diluted with supernatant from uninfected EPC cultures. Test mixtures were kept frozen at –40 °C until assayed.

Laboratory viral infections

Healthy young turbot (1-2 g per fish) were obtained from commercial farms and mantained in 30 L aquaria at 15 °C (30 fish per aquarium) provided with biological filters. To infect the turbot, the water level was reduced to 2 L and 10^8 $TCID_{50}$ of IPNV or VHSV freshly obtained from infected EPC monolayers was added. Only 3 and 4 fish, respectively, died after about a week and no further mortalities were registered after more than 2 months later. Dead turbot were frozen and its kidney homogenates obtained as above and assayed by ELISA 150-fold diluted.

RESULTS

Selection of MAbs

MAbs anti-VHSV and anti IPNV were selected from previous work that were non-competitive, wide range of virus reactivity and had a high titre (Sanz, Coll, 1992a; Babin *et al.*, 1991). MAbs 2C9 for VHSV and 2F12 for IPNV have the highest titres when tested by indirect ELISA with plates coated with their respective purified virus. Conjugation with peroxidase did not reduce their titre (not shown).

Because high-ionic strength buffers and one-step ELISA were required to obtain the highest sensitivity for VHSV (Sanz, Coll, 1992a), anti-IPNV MAbs were selected from those described (Babin *et al.*, 1990; Domínguez *et al.*, 1990, 1991) that were able to work under those conditions. The two MAbs (2A1 and 2F12) performed well in the ELISA and also slighly increased its sensitivity.

To increase sensitivity and decrease the background, the concentration of Tween in the sample dilution buffer was increased from 0.5 g/l to 4 g/l. Both one-step ELISA assays performed well under these new conditions (not shown).

Analytical variables of the one-step ELISA with purified viruses

An example (means and ranges from 3 different ELISA experiments) of the standard curves for the VHS and the IPN viruses binding to the MAbs coated solid-phase is illustrated in Figure 1. The linear part of the assays were between 0.6 to 3.0 absorbance units at 492-620 nm, corresponding to 10 to 200 ng of virus per well. No hook effect was found up to a maximum concentration of virus of 1000 ng/well. The mean absorbance values of the background (in the absence of added virus) varied from 0.05-0.1 ± 25 p. 100 (n = 4). Minimum detection was

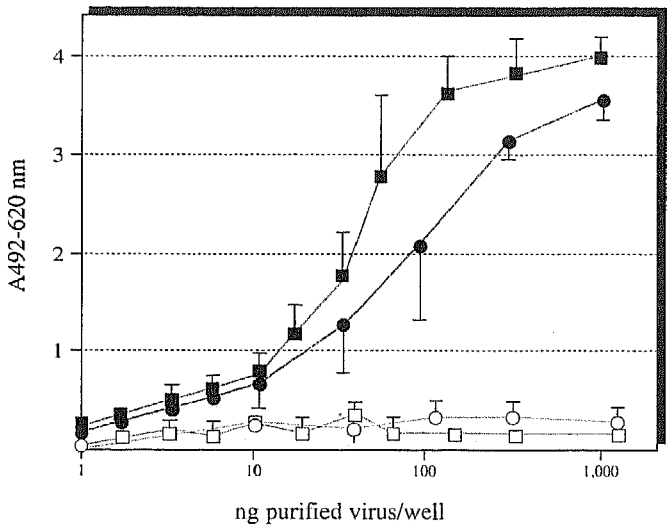

Fig. 1.–Comparative standard curves obtained with purified VHSV (●, ○) and IPNV (□, ■) by the one-step sandwich ELISA
Curvas comparativas standard del ELISA sandwich en un solo paso obtenidas con VSHV (●, ○) y VNPI (□, ■) purificados

The mean of three different experiments and its standard deviations are given in the figure. Plates coated with, ●———●, anti-VHSV 2D5 MAb; ■———■, anti-IPNV 2A1 MAb; ○———○, irrelevant mouse IgG_1; □———□, irrelevant mouse IgG_{2a}
La media y sus desviaciones standard de 3 experimentos diferentes se dan en la figura. Placas tapizadas con: ●———●, AcM anti-VSHV 2D5; ■———■, AcM anti-VNPI 2A1; ○———○, IgG_1 irrelevante de ratón; □———□, IgG_{2a} irrelevante de ratón

defined as the lowest virus concentration that could be detected over the background with 95 p. 100 confidence interval by assuming a normal distribution. Minimum detection corresponded to an OD of 0.07-0.15, which was aproximately 2-4 ng of virus/well, in both cases. Intra-assay coefficient of variation of the absorbance of the standards varied between 3-10 p. 100. Inter-assay ranges were dependent on the virus concentration as shown in Figure 1.

The two MAbs selected were analysed by sandwich ELISA assays with both purified VHSV and IPNV. The data showed that each assay was specific for its own virus whereas the heterologous virus was not recognized.

One-step ELISA of turbot kidney homogenates mixed with viruses

To define the cut-off between negative and positive samples, homogenates of pooled healthy turbot kidney were prepared and assayed by the one-step ELISA. Backgrounds caused by the kidney homogenates were estimated in two ways, first by using plates coated with anti-virus MAbs and healthy kidney homogenates (not shown) and second by using plates coated with non-specific antibodies and virus-containing kidney homogenates (Fig. 2).

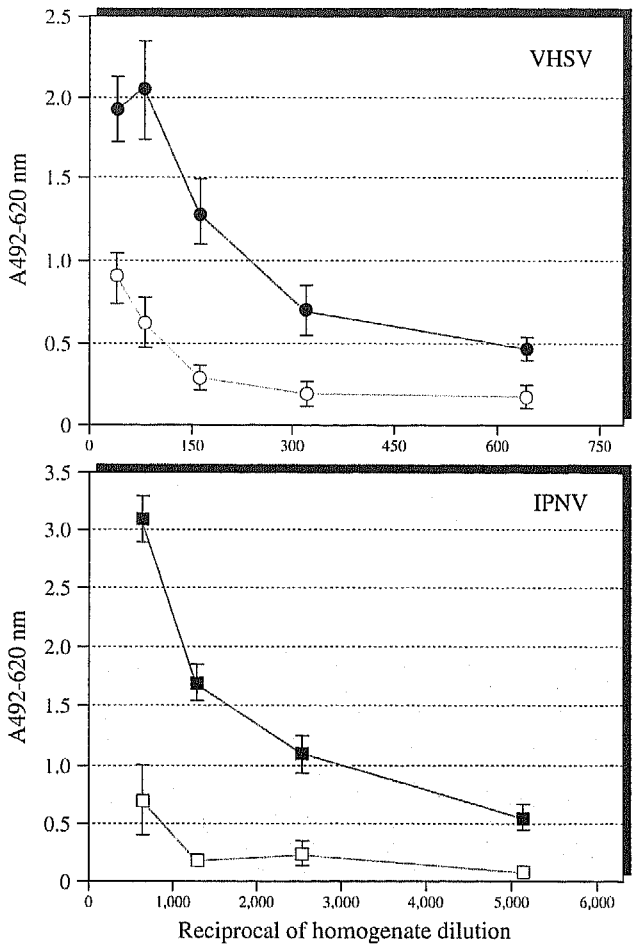

Fig. 2.–Relationship between VHSV and IPNV concentrations in turbot kidney homogenates and absorbances by the one-step sandwich ELISA

Relación entre las concentraciones de VSHV y VNPI en extractos de riñón de rodaballo y absorbancias por ELISA sandwich en un solo paso

VHSV or IPNV were added to turbot kidney homogenates. Samples were tested by one-step sandwich ELISA at different homogenate dilutions in duplicates. Averages and ranges are given in the figure. ●——●, turbot kidney homogenates containing VHSV assayed in plates coated with anti-N 2D5 MAb. ○——○, turbot kidney homogenates containing VHSV assayed in plates coated with irrelevant mouse IgG_1. ■——■, turbot kidney homogenates containing IPNV assayed in plates coated with anti-VP_3 2A1. □——□, turbot kidney homogenates containing IPNV assayed in plates coated with irrelevant mouse IgG_{2a}

VSHV y VNPI se adicionaron a extractos de riñón de rodaballo. Las muestras se ensayaron por ELISA sandwich en un solo paso a varias diluciones del extracto en duplicados. Medias y rangos se dan en la figura. ●——●, *extractos de riñón de rodaballo con VSHV ensayados en placas tapizadas con AcM anti-N 2D5.* ○——○, *extractos de riñón de rodaballo con VSHV ensayados en placas tapizadas con IgG irrelevante de ratón.* ■——■, *extractos de riñón de rodaballo con VNPI ensayados en placas tapizadas con AcM anti-VP · 2A1.* □——□, *extractos de riñón de rodaballo con VNPI ensayados en placas tapizadas con* IgG_{2a} *irrelevantes de ratón*

By using plates coated with anti-virus MAbs, backgrounds obtained with 40-fold diluted healthy turbot kidney homogenates were low (≤ 0.05 p. 100 of the maximal value obtained with virus-containing turbot kidney homogenates).

By using plates coated with non-specific antibodies (irrelevant mouse IgG_1 or IgG_{2a}), the lower the dilution of the homogenate the higher the background. Thus, for a minimum 40-fold dilution of the homogenate (maximal sensitivity), backgrounds obtained for VHSV or IPNV were about 51 p. 100 or 25 p. 100, respectively, of the value obtained with plates coated with anti-virus MAbs (Fig. 2). More than 66000 apparent $TCID_{50}$/ml (150-fold dilution) of VHSV-added turbot kidney homogenates and ≥ 80000 apparent $TCID_{50}$/ml (1250-fold dilution) of IPNV-added turbot kidney homogenates could be considered positives (backgrounds of 22 p. 100 or 11 p. 100 respectively) (Fig. 2).

One-step ELISA of viral infected turbot kidney homogenates

All the few young turbot that die after the laboratory infection (3 turbot after IPNV and 4 turbot after VHSV infection of a total of 30 turbot infected in each case) were found positives (absorbance values ≥ 3-fold its background) by the one-step ELISA.

DISCUSSION

This report describes the use of an unique buffer and protocol for MAbs in one-step ELISA to detect VHSV and IPNV seeded in kidney turbot homogenates. Non-competitive anti-N (VHSV) and anti-VP3 (IPNV) MAbs were selected for the one-step ELISA assay because the N and VP3 proteins are abundant for rhabdoviruses or birnaviruses. The epitopes defined by the MAbs were different (non-competitive) and highly conserved in many VHSV (Sanz *et al.*, 1993) and IPNV (Babin *et al.*, 1991; Domínguez *et al.*, 1991) serotypes. Furthermore, these proteins are structural components found both in the complete virus and in infected cells (Basurco *et al.*, 1991; Deuter, Enzman, 1986; Mc Allister, 1988, Estay *et al.*, 1990).

Attempts to develop ELISA assays for the detection of rhabdoviruses showed that the virus in the cell culture supernatant before any cytopathic effect could be detected. This assays were specific for IHNV or VHSV, but sensitivity was a problem (Dixon, Hill, 1984; Way, Dixon, 1988) until high ionic strength buffers (Sanz, Coll, 1992a) and/or better MAbs were used (Mourton *et al.*, 1992). The high ionic strength buffer disrupts the VHSV nucleocapsid and contributes to lower the background by reducing nonspecific binding. Although no background (≤ 0.05 p. 100) was detected when only turbot kidney homogenates were assayed in wells coated with anti-virus MAbs, there was high background (51 p. 100) when virus-seeded turbot kidney homogenates were added to control plates coated with non-specific antibodies. This could have been due to non-specific binding of the virus to the plates. However, since non-specific binding was absent when turbot kidney homogenates were added with anti-virus antibodies or when purified viruses were added to plates coated with non-specific antibodies (Fig. 1), the background was most likely caused by the turbot tissue-virus complex. That is why the use of control plates coated with non-specific mouse antibodies (IgG_1 and IgG_{2a}) in parallel ELISAs is required to estimate posible false positives. The extent of

false positive signals will depend, on the final dilution of the turbot kidney homogenate (Fig. 2).

The 100 µl sample requirement is especially desirable for testing individual turbot fingerlings. Attributes of these ELISAS include, only one-step, stable reagents (Martinez, Coll, 1988), addition of phenol red to allow visualization and monitoring of pH, using microtitre plates divided in rows which adapt to the number of assays needed, low background peroxidase-o-phenylenediamine buffer with added merthiolate for increasing stability (Coll, 1989), use of selected MAbs easily produced as mice ascites, and simple conjugation to peroxidase of the MAbs selected (Coll, 1987b). These characteristics makes the new method scalable and reproducible (Coll, 1991). Multiple homogenizers made in 96 well plates could be used to obtain individual homogenates (French-Constant, Devonshire, 1987). Individual homogenates can be transferred with a multichannel pippete to the ELISA solid-phase. The time spent to examine 40 samples could be $\leq$ 4 hours. Entire fish could be frozen and kept at –20 °C before performing the ELISA.

Since the ELISA gave a strong visual positive reaction when turbot extracts mixed with cell culture-grown virus were used, it might be possible to use it for diagnosis of the clinical disease in the laboratory. Preliminarly attempts, although positive were not stadistically significant due to the low mortalities obtained with the laboratory viral infections of young turbot. The ELISA, however might not be as sensitive (Sanz, Coll, 1992b) as cell culture for detecting virus infected turbot and therefore its use in routine diagnostic of diseased turbot cannot yet be advocated. The method described here has to be evaluated against other methods, particularly with direct isolation to stablish its potential value. Perhaps, in the future, a panel of different ELISA assays (IPNV, VHSV and IHNV) could be used to provide a first rapid detection and identification of these diseases in the field (Ristow, Arnzen, 1989, 1991).

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RESUMEN

ELISA en un paso y protocolo único para detectar los virus de la necrosis pancreática infecciosa y de la septicemia hemorrágica viral en rodaballo

Se ha desarrollado un único protocolo para seleccionar y usar anticuerpos monoclonales (AcM) para dos ELISAS tipo sandwich en un solo paso para detectar el virus de la necrosis pancreática infecciosa (NPI) y de la septicemia hemorrágica viral (SHV) en extractos de riñón de rodaballo. Para la mayor sensibilidad, los extractos de riñón de rodaballo mezclados con los virus pueden ser diluidos $\geq$ 40 veces con tampones de alta fuerza iónica y ensayados para la presencia de nucleoproteínas N/Nx del SHV o proteína estructural VP3 del NPI. Los resultados preliminares muestran que el ensayo es capaz de detectar ambos virus en infecciones experimentales de jóvenes rodaballos.

PALABRAS CLAVE: ELISA
Anticuerpos monoclonales
Virus
Rodaballo

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