Application of 3 techniques for diagnosing birnavirus infection in turbot

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Vet Res (1995) 26, 493-498

Application of 3 techniques for diagnosing birnavirus infection in turbot

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Summary — Three procedures for avoiding viral amplification by cell culture were evaluated for the diagnosis of birnavirus infections of turbot *(Scophthalmus maximus)* tissues. Immunodot assay using monoclonal and polyclonal antibodies was not satisfactory as a detection system because of false positive reactions. Although immunofluorescent assay of liver and kidney smears was an adequate method for a rapid diagnosis of the infection, blood smears from infected fish did not show higher levels of fluorescence than those from uninfected fish. However, the detection of birnavirus by polymerase chain reaction (PCR) amplification directly in fish tissues was successful and seems to be a promising system for the diagnosis of fish birnavirus.

birnavirus / IPNV / fish virus diagnosis / turbot

Résumé — Comparaison de 3 techniques pour le diagnostic du birnavirus du turbot. *Trois méthodes ne faisant pas appel à la multiplication du virus en culture cellulaire ont été évaluées pour le diagnostic des infections à birnavirus dans les tissus du turbot* (Scophthalmus maximus). *La technique de l'immunodot avec des anticorps monoclonaux ou polyclonaux n'a pas donné satisfaction comme système de détection à cause des fausses réactions positives. Bien que l'immunofluorescence sur des frottis de rate et de rein ait été une méthode adéquate pour un diagnostic rapide de l'infection, les frottis sanguins de poissons infectés n'ont pas montré plus de fluorescence que ceux de poissons sains. Cependant, la détection du birnavirus par amplification en chaîne par polymérase (PCR) pratiquée directement sur les tissus de poisson a été couronnée de succès et semble un moyen prometteur pour le diagnostic des birnavirus de poisson.*

birnavirus / virus de la nécrose pancréatique infectieuse / diagnostic du virus de poisson / turbot

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INTRODUCTION

Viral diseases have become a serious problem in world aquaculture. Infectious pancreatic necrosis virus (IPNV) is the etiological agent of a contagious disease that produces high mortalities in young salmonids. There are many related viruses with different antigenic and biochemical characteristics. These viruses belong to the *Birnaviridae* family whose members are characterized by a bisegmented, double-stranded RNA genome (Wolf, 1988).

The control of birnavirus infections in aquatic animals requires sensitive, safe and rapid assays for detection and identification of the viral pathogen. Recent isolations of these viruses from other non-salmonid species imply a risk for new promising fish cultures, both freshwater and marine. This is the case for turbot *(Scophthalmus maximus),* which is being successfully cultured in Europe, mainly in Spain and Norway, with an important market and high economic value (Mortensen *et al,* 1993; Novoa *et al,* 1993).

Isolation of virus by inoculation of cell culture with fish samples has been, until now, the first step in fish disease diagnostic laboratories for diagnosing such etiological agents (Amos, 1985); however, this system requires time, and is relatively expensive and tedious. Once the viral agent has been isolated, the identification is usually made by seroneutralization with polyclonal antibodies. Several other serological assays have been adapted for this viral group, including immunofluorescent and immunoperoxidase staining of infected cell monolayers and a complement fixation test (Wolf, 1988). More recently, immunoenzymatic assays, such as immunodot and ELISA, have been accepted as sensitive and rapid systems for the identification of birnaviruses (Nicholson and Caswell, 1982; Dixon and Hill, 1983; Hattori *et al,* 1984; Rodak *et al,* 1988; Hsu *et al,* 1989; Dominguez *et al,* 1990). When monoclonal antibodies are used, a rapid identification or a presumptive serotyping of the virus can be made, depending on the specificity of the antibody (Caswell-Reno *et al,* 1986, 1989). Several attempts have been made to find methods which do not require lethal sampling. This is very important in brood stocks or in species with high economic value (Rodriguez *et al,* 1991). More recently, polymerase chain reaction (PCR) assays have been used to identify some specific strains of aquatic birnaviruses (McAllister *et al,* 1991; Lee *et al,* 1994; Blake *et al,* 1995).

The aim of this work was to find a suitable method for the rapid diagnosis and identification of these viruses, which would help limit the spread of birnavirus infections and the consequent economic losses. We investigated 3 techniques for diagnosing these important fish infections directly in the fish, without the need for cell culture inoculation, in order to obtain a quick presumptive diagnosis.

MATERIALS AND METHODS

Viral strains

The birnavirus strain, TB-306, isolated from turbot *(Scophthalmus maximus)* (Novoa *et al,* 1993) was used. The virus stock was prepared in the chinook salmon embryo cell line (CHSE-214). When the cytopathic effect (CPE) was extensive, the cells were harvested and centrifuged to eliminate cell debris. The virus stock was titered (Reed and Müench, 1938) and stored at −70°C until use.

Experimental infection

Turbot with a mean weight of 10 g were inoculated intraperitoneally with 50 µl of a viral suspension of the birnavirus strain TB-306 with a

titer of 10^8 $TCID_{50}$/ml. The controls were treated as the infected fish and were inoculated with cell culture medium. Fish were maintained in 25 l tanks using recirculated sea water with aeration. After 10 d, fish were sacrificed and dissected. Samples of liver and kidney were taken for virological assays which were processed following standard procedures (Amos, 1985). Titrations of the virus were made in 96 multiwell plates of CHSE-214 cell cultures and the $TCID_{50}$ titers determined according to the method of Reed and Müench (1938). In addition, smears of liver, kidney and blood were made, fixed with acetone at 4°C for 10 min, and frozen at –20°C until use.

Immunodot assay

A polyvalent, polyclonal antibody (PAb), prepared in rabbit against IPNV, was used. In addition, 2 monoclonal antibodies (MAb) (AS-1 and E-5), which recognize a common epitope of birnavirus serogroup A (Caswell-Reno *et al,* 1989), were used. Briefly, 2-fold dilutions of the tissue samples were adsorbed onto a hydrated nitrocellulose membrane and were incubated for 1 h at room temperature. Two hundred microlitres of membrane-blocking solution (1% BSA in TBS or skim milk) was added to each well for 1 h. The wells were washed with TBS and 100 µl of the antibody (rabbit PAb or mouse MAbs), undiluted or diluted 1:2, were incubated for 1 h. Then, 100 µl of mouse or rabbit anti IgG peroxidase-conjugated antibody were added for 1 h. The 4-Cl-1-naphthol solution was added until the dots turned purple.

Immunofluorescence assay

The smears of liver, kidney and blood were treated for 1 h with a polyvalent antiserum against IPNV (1:100 dilution) or with the MAbs specific for serogroup A of birnaviruses (AS-1 and E5) (1:100 dilution). After this, 2 washes with PBS were made and a dilution of 1:250 of conjugate (IgG-FITC anti-mouse or anti-rabbit, Sigma) was added for 1 h. The preparations were washed with PBS, stained for 5 min with Evans' blue (Sigma) and mounted with low fluorescence buffered glycerine (BioMérieux) for observation in a microscope equipped with epifluorescence (Nikon, Optiphot 2).

PCR amplification

RNA was extracted by phenol/chloroform from 100 µl of kidney homogenates. Tissue homogenates were digested in lysis buffer containing protein kinase for 3 h and then extracted with an equal volume of phenol/chloroform followed by NaAc/ethanol precipitation at –20°C overnight. After washing the pellet with 70% EtOH, the samples were centrifuged and the pellets dried. The preparations were heated at 95°C for 5 min to denature the viral dsRNA and used as a template in cDNA synthesis with the Gene Amp RNA PCR-kit (Perkin Elmer). The amplification was performed in a DNA thermal cycler (MJ Research Inc) and 35 amplification cycles (94°C for 30 s, 58°C for 30 s and 92°C for 1 min) were performed. A pair of primers (Blake *et al,* 1995) developed from the published cDNA sequences of Jasper and N1 strains (Duncan and Dobos, 1986; Havarstein *et al,* 1990) were used. These primers directed the synthesis of a 173 bp segment of cDNA within the genomic region encoding the VP3 protein. The amplified products were analyzed for purity and size by electrophoresis in 1.2% agarose gels at 60 V for 4 h. The gels were stained with ethidium bromide and visualized with a UV light source.

RESULTS AND DISCUSSION

No successful results were obtained in the direct serological detection of birnavirus from the organ homogenates by the immunodot assay. False positive reactions were found in uninfected organs from fish injected with MEM. Saturation of the matrix binding sites resulting from the high protein concentration of turbot homogenates was observed in both cases which interfered with the bindings of both polyclonal and monoclonal antibodies. Several dilutions of the tissue samples were assayed without any improvement in the results.

The immunodot assay has the advantages of simplicity, ease of interpretation and economy of time and reagents (McAllister and Schill, 1986) and has been successfully used in detecting birnavirus from

cell culture supernatants (McAllister and Schill, 1986; Hsu *et al,* 1989). There are some reports of positive results obtained when this technique is applied to detect birnavirus *in vivo* in salmonid species (Hsu *et al,* 1989; Babin *et al,* 1991). In the present study, the appearance of false positives (reaction with the uninfected tissue) constituted an important limitation. Other authors have pointed out the presence of non-specific reactions with fish tissues (Hattori *et al,* 1984; Rodak *et al,* 1988; Ross *et al,* 1991). One reason is that the matrix was saturated by the high content of protein in fish homogenates (McAllister and Schill, 1986). Therefore, this technique could be used after a cell culture amplification, but not directly with clinical specimens.

There were clear differences between smears from infected or uninfected fish when the immunofluorescence assay was performed. Smears from infected liver and kidney showed a higher intensity of fluorescence than those from control turbot, which present a background with only weak fluorescence. However, it was always not easy to distinguish blood smears from infected and uninfected fish. The results obtained with polyclonal or monoclonal antibodies were similar.

The immunofluorescence assay has been extensively applied for *in vivo* detection of viral agents in cell cultures and fish tissues (Sanz and Coll, 1992). In addition, Swanson and Gillespie (1982) used it in frozen tissue sections from infected fish with fluorescence found in pancreas, liver and kidney. The results of our work show that the use of immunofluorescent assays with tissue smears can be a useful system to diagnose the infection. However, interpretation of the results requires experience and considerable judgment and can be difficult.

PCR amplification proved to be the most useful method for diagnosing birnavirus infections in turbot kidney tissue. PCR amplification products were clearly detected in agarose gels (fig 1). A band corresponding to the expected 173 bp amplification fragment appeared only in the kidney homogenates from infected turbot, but it was never present in the homogenates from control fish.

In this study, PCR amplification proved to be a promising procedure to directly detect birnaviruses in fish tissues. This technique is widely used in human and animal diagnostics, but in the fish diseases field it is not very common. Until now, there are few reports of the application of PCR to detect viral infections of fish (Arakawa *et al,* 1990; Rimstad *et al,* 1990; Lee *et al,* 1994; Blake *et al,* 1995). Our results show that the PCR

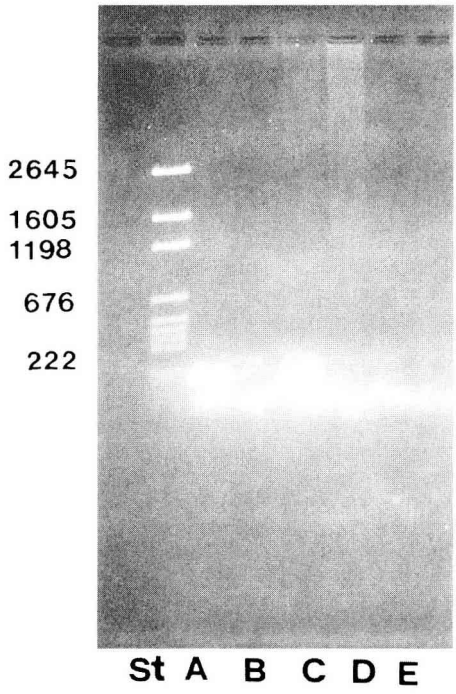

Fig 1. Agarose gel electrophoresis of PCR products from turbot kidney homogenates. Lanes A and C correspond to the reverse-PCR product obtained from infected kidneys. Lanes B, D and E correspond to uninfected kidneys, with no 173 bp band. St: standard of molecular weight (pGEM DNA marker, Promega).

assay is a simple, fast and highly sensitive method for the diagnosis of aquatic birnaviruses, especially for a large number of samples. Although costs should be evaluated, we propose this as a suitable technique for diagnosing birnavirus infections in aquatic animals, and, specifically, for marine species such as turbot.

Most of the various techniques used for the diagnosis of birnavirus infections in aquatic animals require inoculation of cell cultures with fish tissue homogenates. However, most fish cell lines currently used in fish virology laboratories are derived from freshwater species. This fact could be a limiting factor when virological assays are performed from marine fish. Host specificity could limit the detection of viruses in the marine environment, despite the increasing number of marine fish cell lines established (Nicholson *et al,* 1987; Tung *et al,* 1991; Fernandez *et al,* 1993; Fernandez-Puentes *et al,* 1993). More effort should be placed on this subject and alternative techniques should be developed to improve the speed and specificity of viral detection.

ACKNOWLEDGMENTS

We are grateful to A Ledo for her cooperation. This work was supported by Grant AGF93-0769-C02-02 of the Comisión Interministerial de Ciencia y Tecnología (CICYT). B Novoa acknowledges the Ministerio de Educación y Ciencia (Spain) for a research fellowship.

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