

A genetic probe for identification of the turbot aquareovirus in infected cell cultures

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ABSTRACT: A nucleic acid hybridization assay has been developed that can detect small amounts of the turbot aquareovirus (TRV) in infected cells. Complementary DNAs were synthesized from the dsRNA genome segments of TRV and used to generate a large number of recombinant plasmids. Plasmids corresponding to genomic segments 1 through 8 were identified by Northern blot analysis. A randomly selected clone, Clone 66, hybridizing to genome Segment 8, showed high specificity hybridizing only RNA from TRV, and not with the RNAs of 4 other aquareoviruses, or with RNA extracted from infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV) or with uninfected cells. A ³²P-labeled probe was able to detect as little as 50 ng of TRV purified virus dsRNA. Time course experiments indicated that TRV RNA can be detected in infected cells 96 h post-inoculation, a time when cytopathic effect is still not evident. These results indicate that the dot blot assay described here could be used as an effective diagnostic tool for the detection of TRV infections.

INTRODUCTION

Over the last 15 years, an increasing number of viruses with characteristics of the family Reoviridae have been isolated from aquatic animals, including crustacea, shellfish and fish from different geographic areas and environments (Hetrick et al. 1992). These viruses produce syncytia as a typical cytopathic effect (CPE) in susceptible fish cell lines. The virions are 70 to 75 nm in diameter and consist of a double-layered capsid containing a genome composed of 11 segments of doublestranded RNA (ds RNA). These viruses share some characteristics with the genera *Rotavirus* and *Orthoreovirus* but based on their host range, optimal growth temperature, RNA electrophoretic pattern, RNA-RNA hybridization studies and serological tests, they have been placed in the new genus *Aquareovirus* in the family Reoviridae (Francki et al. 1991).

Although most of these viruses were isolated during routine examination of apparently healthy fish (Meyers 1979, Winton et al. 1981, 1989, Ahne & Kölbl 1987,

Samal et al. 1991), some of them were isolated from fish suffering a mixed bacterial and viral infection (Lupiani et al. 1989, Baya et al. 1990), or from fish populations undergoing chronic or large mortalities (Plumb et al. 1979, Amend et al. 1984, Chen & Jiang 1984, Hsu et al. 1989, Marshall et al. 1990, Varner & Lewis 1991, Jiang et al. 1991). In 1987, an aquareovirus (TRV) was isolated from cultured turbot during a disease outbreak in northwest Spain (Lupiani et al. 1989). During the outbreak, there was a continuous but low mortality, with a cumulative loss of 4 % of the affected population. A bacterium of the genus *Vibrio* was isolated from the same fish. Treatment of the affected fish with an antibiotic to which the bacterium was sensitive only temporarily suppressed mortality which suggested a possible role of TRV in the development of the disease.

Since vaccines to prevent aquareovirus infections are not available at this time, the best control measure is to prevent the introduction of these viruses into aquaculture facilities. Currently, detection of aquareoviruses requires virus isolation and serological identification. Since aquareoviruses grow relatively slowly (10 to 14 d) there is a need for a more rapid detection method.

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Nucleic acid hybridization techniques have been employed for the identification of many human and animal viruses (Kulski & Norval 1985, Viscidi & Yolken 1987, Rimstad et al. 1990). We describe here the development of such an assay for the detection of TRV infections in fish. This involved the synthesis of cDNA clones of the TRV strain of aquareovirus so that the probe can be produced in large quantities at reasonably low cost.

MATERIALS AND METHODS

Viruses and cells. Five strains of aquareovirus were used: TRV, isolated from turbot *Scophthalmus maximus* in Spain (Lupiani et al. 1989), SBR, isolated from striped bass *Morone saxatilis* collected in the Chesapeake Bay, USA (Baya et al. 1990); SRV isolated from smelt *Osmerus mordax* in Canada (Marshall et al. 1990); and HBR and ASV isolated from the sex products of different populations of Atlantic salmon *Salmo salar* in the USA and Canada, respectively (Samal et al. 1991). Two other fish viruses, infectious pancreatic necrosis virus (IPNV), a birnavirus, and infectious hematopoietic necrosis virus (IHNV), a rhabdovirus, were used as controls to determine the specificity of the probes. All viruses were propagated at 15 °C in chinook salmon embryo cells (CHSE-214) grown in minimum essential medium (MEM) supplemented with 10 % fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 2.5 µg ml⁻¹ fungizone.

Virus purification and extraction of double-stranded RNA. Virus particles were purified as previously described (Ramig et al. 1977, Samal et al. 1990) using PEG precipitation, freon extraction and sucrose gradient sedimentation. Purified virus was resuspended in 1X SSC and stored at 4 °C until used. The dsRNAs were extracted from purified virus with phenol:chloroform (1:1), chloroform and ether, and precipitated with ethanol and sodium acetate.

Cloning of the genomic segments for preparation of diagnostic probe. The method of Cashdollar et al. (1982) was used with slight modifications for cloning TRV dsRNA. Briefly, approximately 5 µg of dsRNA was denatured in 10 mM methylmercury hydroxide at room temperature for 10 min. Excess methylmercury hydroxide was quenched by the addition of 140 mM β-mercaptoethanol. Random hexamers (Pharmacia, Piscataway, NJ, USA) were used to prime the first strand reaction. Denatured viral RNA (5 µg) and random primers (5 µg) were mixed in a reaction buffer (50 mM Tris HCl pH 8.3, 10 mM MgCl₂, 10 mM dithiothreitol, 4 mM sodiumpyrophosphate, 1.25 mM of each of the deoxynucleotide triphosphates) and 100 U of avian myeloblastosis virus reverse transcrip-

tase (AMV) was added and the mixture incubated for 1 h at 42 °C. The reaction products were extracted with phenol:chloroform (1:1) and precipitated with ethanol and ammonium acetate. Second-strand synthesis was carried out as described by Gubler & Hoffman (1983) using RNase H and *Escherichia coli* DNA polymerase I. The double-stranded DNA was blunt-ended with T4 DNA polymerase, and *EcoRI* linkers (Promega Biotech) were ligated to the blunt-ended cDNA with T4 DNA ligase (Sambrook et al. 1989). A sepharose CL4B column (Pharmacia) was used to separate *EcoRI*-ended cDNA from free linkers. The cDNA was then phosphorylated using T4 polynucleotide kinase and ligated to *EcoRI*-digested plasmid, pUC19. This preparation was used to transform *E. coli* JM 109 cells (Hanahan 1985) and the transformed mixture was plated on bacterial culture plates containing ampicillin, IPTG, and X-Gal. Bacteria containing recombinant plasmids were identified by ampicillin resistance and white colonies. Plasmids were isolated from randomly selected colonies by the method described by Birnboim & Doly (1979). Plasmids were digested with *EcoRI* and examined by agarose gel electrophoresis to confirm the presence and determine the size of the inserts.

Northern blot hybridization. Northern blot hybridization (Alwine et al. 1979) was used to determine the specificity of cDNA inserts for each of the 11 genome segments of TRV. Briefly, probes were prepared by excising and extracting cDNA inserts from agarose and radiolabeling by random-priming (Feinberg & Vogelstein 1983). The TRV genomic dsRNA segments were electrophoresed in polyacrylamide gels, transferred to Gene Screen Plus membrane by electroblotting and hybridized with radiolabeled cDNA probes. The dsRNA genomic segment reacting with each probe was determined by comparison with the 11 TRV dsRNA segments that were 3'-end-labeled using [5'-³²P]pCp (England & Uhlenbeck 1978). Prehybridization and hybridization was carried out at 42 °C as described by Bodkin & Knudson (1985).

Isolation of total RNA from virus-infected cells. Confluent monolayers of CHSE-214 cells grown in 25 cm² cell culture flasks were infected with the 5 aquareovirus strains, IPNV and IHNV at a multiplicity of infection (MOI) of 0.01 and 0.1. After 1 h of adsorption, the monolayers were washed with PBS and maintenance medium (MEM plus 2 % FBS) was added. When CPE was evident, the cells were removed from the culture flask and resuspended in 1 ml of TNE buffer (10 mM Tris HCl pH 7.4, 1 mM EDTA, 150 mM NaCl). The suspension was brought to 0.5 % Nonidet P-40 and kept on ice for 10 min. Nuclei were pelleted at 14000 × *g* for 5 min and the supernatant was transferred to a new tube. SDS (0.1 % final concentration)

and proteinase K ($200 \mu\text{g ml}^{-1}$) were added to the supernatant and the solution was incubated at 45°C for 1 h. The mixture was extracted first with phenol:chloroform and then with chloroform alone. Total RNA was precipitated with sodium acetate and ethanol and the pellet was resuspended in TE buffer (10 mM Tris pH 7.4, 1 mM EDTA).

Time course experiments. Time course experiments were conducted to determine the minimum incubation period required for production of detectable levels of TRV RNA. Replicate monolayers of CHSE-214 cells were infected with TRV at MOIs of 0.01 and 0.1. After 1 h, the monolayers were washed with PBS to remove unadsorbed virus and maintenance medium was added. At various times post-infection, cells were removed and RNAs extracted for blotting as described above. RNAs from mock-infected cells and the heterologous viruses, IPNV and IHNV, were used as negative controls.

Dot-blot hybridization of dsRNA. RNA extracted from infected cell cultures was denatured by the addition of 10 mM methylmercury hydroxide and then spotted onto a Gene Screen Plus membrane using a 96-well filtration unit. Membranes containing viral RNA were baked at 80°C for 30 min and then soaked in a prehybridization solution for 2 h at 42°C using Hybrisol I (Oncor) (50% formamide). Labeled cDNA probes were denatured by heating at 100°C for 5 min and added to the prehybridization mixture. After overnight hybridization at 42°C , the membranes were washed at room temperature in $1\times\text{SSC}$ containing 0.1% SDS and then at 58°C in $0.1\times\text{SSC}$ containing 0.1% SDS for 1 h before autoradiography.

RESULTS

Synthesis of TRV virus cDNA clones

More than 1000 ampicillin-resistant colonies were obtained from the cloning procedure used. All white colonies tested by enzymatic digestion analysis contained plasmids with cDNA inserts. The size of the different

inserts was determined on 1% agarose gels and ranged from 250 to 1800 nucleotides. Recombinant plasmids were identified by hybridization of radio-labeled inserts to a blot of TRV virus dsRNA segments separated by SDS-PAGE. No clones corresponding to Segments 9, 10 and 11 were detected, although many clones corresponding to the other segments were found. Among these, Clone 8 reacted with Segment 2, Clone 19 with Segment 3, Clone 58 with both Segments 2 and 3, Clone 66 with Segment 8, and Clone 67 with Segment 6 (Fig. 1).

Specificity of probes from TRV aquareovirus cDNA clones

The specificity of one randomly selected clone, Clone 66 corresponding to Segment 8, was examined by hybridization to dot blots of dsRNA extracted from 5 purified aquareovirus strains (TRV, SBR, HBR, ASV and SRV) as well as to dot blots of total RNA extracted

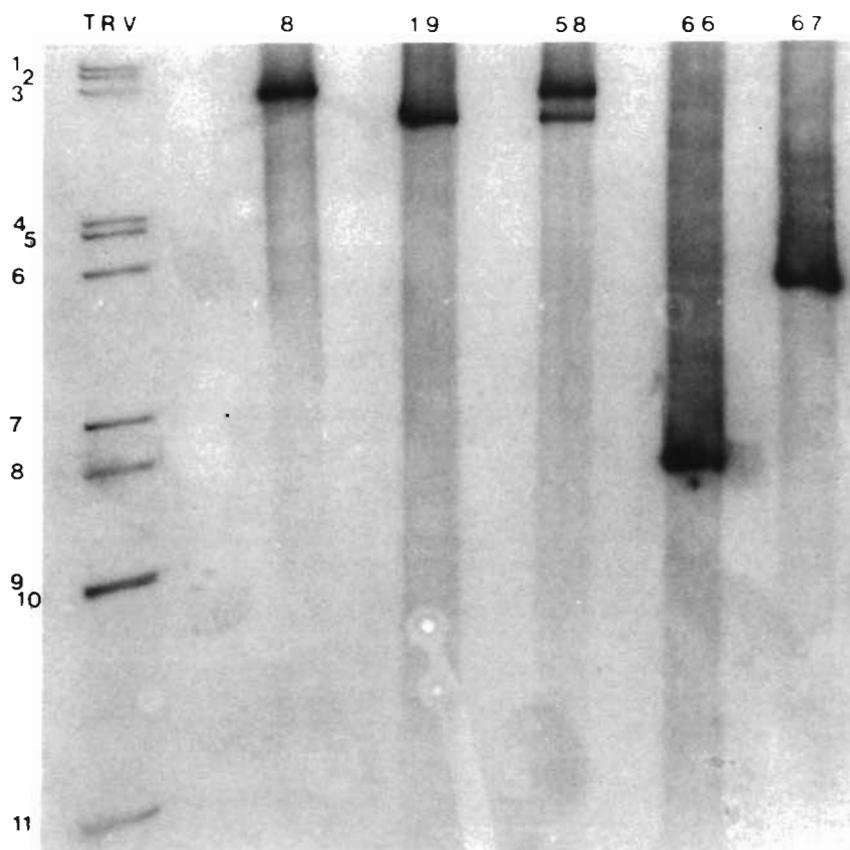
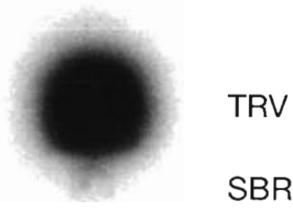


Fig. 1. Identification of TRV cDNA clones by Northern blot analysis. TRV virus dsRNA was electrophoresed in a 6% SDS-PAGE gel and the RNA was electrophoretically transferred to a Gene Screen Plus membrane. cDNA inserts (numbered lanes) were labeled with ^{32}P by random-priming and each insert was hybridized with a strip of the membrane containing all 11 dsRNA segments. The positions of the 11 genome segments are shown in the lane labeled TRV



TRV
SBR
HBR
ASV
SRV

Fig. 2. Autoradiography of the hybridization of ³²P-labeled TRV virus-specific cDNA Clone 66 corresponding to Segment 8, with the dsRNAs of 5 aquareovirus strains. Purified dsRNA was denatured with 10 mM methyl-mercury hydroxide and immobilized onto a nylon membrane. The cDNA clone was labeled with ³²P by the random-primed method and hybridized with the membrane containing the denatured RNA

from cell cultures infected with the 5 strains of aquareovirus, or with IPNV, IHNV, and mock-infected cells. Clone 66 hybridized well with dsRNA extracted from purified TRV (Fig. 2) and with the RNA extracted from TRV-infected cells (Fig. 3), but it did not hybridize with the RNAs of the other aquareoviruses, or with IPNV, IHNV or mock-infected cells.

Sensitivity of the Clone 66 probe

Time-course experiments demonstrated that the Clone 66 probe could detect the presence of TRV RNA in infected cells as early as 96 h post-infection when CPE was still not evident (Fig. 4). The sensitivity of the cDNA probe made from Clone 66 was determined by hybridization with different amounts of dsRNA extracted from purified TRV. Clone 66 detected as little as 50 ng of genomic viral RNA in dot blot hybridization studies (Fig. 5).

DISCUSSION

In the present study, we have described the cloning of cDNA inserts made from the dsRNA genome segments of TRV aquareovirus. A large number of recombinant plasmids were obtained using the cloning procedure described. Plasmids corresponding to genomic Segments 1 through 8 were identified by Northern blot analysis. No recombinant plasmids corresponding to Segments 9 to 11 were detected, probably because they were lost during the RNA purification process due to their small size. The size of the different cDNA inserts ranged from 250 to 1800 nucleotides.

Clone 66, hybridizing to the TRV genome Segment 8, was randomly selected and its specificity and sensitivity were tested for use as a potential probe for diag-

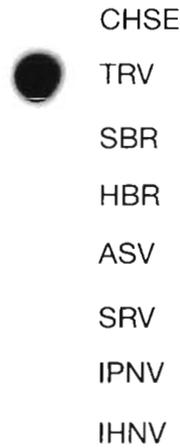


Fig. 3. Detection of TRV virus RNA in infected cells. CHSE-214 cells were infected with the 5 aquareovirus strains, IPNV, and IHNV. Total RNA was extracted from CHSE-214 infected and uninfected cells 6 d post-inoculation, denatured, blotted onto a nylon membrane as described before and hybridized with a ³²P-labeled probe prepared from Clone 66 cDNA

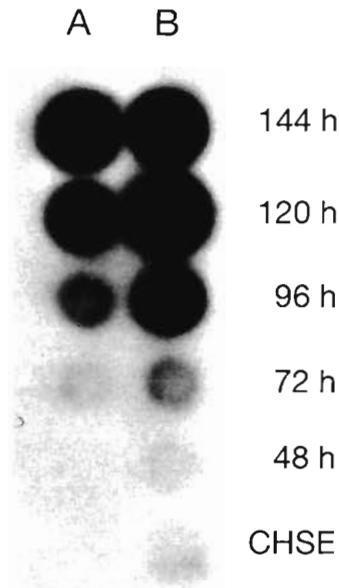


Fig. 4. Time-course experiment of TRV RNA replication. CHSE-214 cells were infected with TRV at an MOI of 0.01 (Row A) and 0.1 (Row B). Total RNA was extracted at 24, 48, 72, 96, 120 and 144 h post-infection. Total RNA was denatured, spotted onto a nylon membrane and hybridized with a ³²P-labeled probe prepared from Clone 66 as previously described

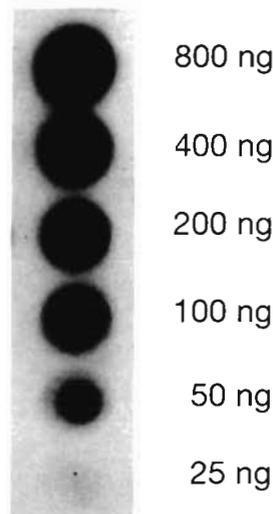


Fig. 5. Sensitivity of Clone 66 for the detection of TRV RNA. Different amounts of RNA extracted from purified TRV virus were spotted onto a nylon membrane and hybridized with a ³²P-labeled probe prepared from Clone 66

nostic purposes. Clone 66 was shown to be highly specific, hybridizing only to RNA from TRV, and not with the RNAs of other aquareovirus strains tested (SBR, HBR, ASV and SRV), or with IPNV, IHNV or uninfected cells. These results indicate that the dot blot assay described here could be used as an effective diagnostic tool for the detection of TRV infections. Experiments are in progress to determine the specificity of the probe in tissue samples of TRV-infected fish and to study the pathogenesis of TRV infections. The specificity of the TRV cDNA probe confirms our previous observations that the TRV isolate is genetically different from other North American aquareovirus isolates (Samal et al. 1991). The specificity of the probe also provides an easy way to determine the presence of other aquareovirus strains genetically similar to TRV and hence to study the genetic relatedness among different aquareovirus isolates.

The cDNA probe was able to detect 50 ng of TRV dsRNA. The sensitivity reported for rotavirus probes in this type of assays is in the range of 0.5 to 5 ng of RNA (Dimitrov et al. 1985, Eiden & Yolken 1987). We are attempting to improve the sensitivity of the cDNA probe by increasing the specific activity of the cDNA and by using highly specific RNA probes as described by Kreig & Melton (1984). Our cDNA probe should also be useful in determining the distribution of TRV in natural fish populations and for studying the structure and function of the TRV genes.

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