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Research Completion Report*

Diagnosis of the Disease Agent in Epizootic Epithelial Disease

by

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COMPLETION REPORT

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Introduction

Epizootic Epithelial Disease (EED) is a disease that has hindered production of lake trout, *Salvelinus namaycush*, at Great Lakes region fish hatcheries. The apparent cause of the disease is a virus tentatively identified as a herpesvirus and named Epizootic Epithelial Disease Virus (EEDV) (Bradley, et al. 1989). Thus far no easily utilizable diagnostic scheme has been developed for EEDV. Density gradient centrifugation followed by electron microscopic visualization has been the only reliable means of viral identification. However, these techniques are cumbersome and not readily applicable to screening large numbers of samples. Development of a simplified detection scheme would be of distinct benefit to the fresh water fish industry in the Great Lakes area. An obvious choice for herpesvirus detection is to experimentally infect tissue culture cells and monitor cell damage or death. Despite intensive efforts including development of a line of lake trout cells, thus far the EEDV has been recalcitrant to observation by these methods (Joe Marcino, personal communication; Paul Bowser, personal communication).

Based on previous success with other herpesviruses (Wise, et al. 1985), it was proposed that nucleic acid based detection schemes might be useful in the case of EEDV. One approach that has been extensively used is produce a DNA probe against the genome of a virus. DNA from a fish suspected of harboring an EEDV infection would be purified, restriction digested, subjected to

electrophoresis, Southern transferred to a membrane, and finally probed with a radioactively labeled DNA probe specific for some viral sequence (Wise and Boyle, 1985). A positive hybridization would indicate presence of viral sequences in the fish. An alternate approach would require knowledge of some sequence information about the viral DNA. Oligonucleotides would be constructed to ends of a particular viral sequence, and the purified fish DNA would be subjected to Polymerase Chain Reaction (Saiki et al. 1988). This protocol has the advantage of much greater sensitivity than probing. It is also simpler and faster. It too has been applied to detection of herpesviruses (Blackwell and Boyle, 1991).

Materials and Methods

Infected fish

Lake trout showing symptoms of EEDV infection were generously provided by Dr. Sue Marcquenski of the Wisconsin Department of Natural Resources.

DNA Purification from Tissue

Tissue from fish was homogenized in phosphate buffered saline, pH 7.2 and clarified by centrifugation at 10,000 x g for 30 min. The supernatant was passed through a 450 nm membrane filter for further clarification. This was then mixed with 7% polyethylene glycol 6000, 0.5M NaCl and kept overnight at 4°C. A pellet was collected by centrifugation at 8000 x g for 1 hr. The pellet was

resuspended in a minimal volume of 10mM TrisHCl, 1mM EDTA, pH 7.2 (TE).

At this point the sample was considered to be a viral suspension. Viral DNA was purified according to the methods outlined in Wise and Boyle, 1985.

DNA Purification from EEDV

Gradient fractions known to be positive for EEDV were kindly provided by Dr. Phil McAllister of the National Fish Health Research Laboratory.

The samples were dialyzed into TE and purified according to Wise and Boyle, 1985.

Restriction Digestion

All purified DNA samples were digested with the restriction enzyme EcoRI (Promega) overnight and subjected to agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized with a short wave UV light.

Shotgun Cloning

Digested DNA was ligated with EcoRI-digested plasmid pUC8 (Messing and Vierra, 1982). The resultant recombinants were transformed into E. coli JM101. The *E. coli* was subsequently screened for the presence of recombinant plasmids by using the metabolite X-gal.

Thymidine Kinase Sequence Comparisons

Genbank was searched for sequences of thymidine kinase genes from herpesviruses using the DNASIS software package (Hitachi). The six sequences found were screened for conserved regions. Oligonucleotides were subsequently produced for the regions identified.

Oligonucleotide Probing

The conserved oligonucleotides were radioactively labeled by addition of multiple alpha-^{32}P cytidines to their 3′ end (Church and Kieffer-Higgins, 1988). Channel Catfish Virus (CCV) DNA was digested with EcoRI or Hae III, electrophoresed, and Southern blotted. It was then probed with the radiolabeled oligonucleotides. Hybridization was overnight. The blot was washed and exposed to X-ray film (XOMAT AR) at -70°C.

Results and Discussion

Attempts at DNA Purification

Our initial plans called for the development of a nucleic acid probe specific for EEDV. Efforts aimed at purification of viral particles from fish tissue were not reproducibly successful. Routinely, tissue from 20 fish yielded nothing. However, an occasional preparation seemed to yield a DNA band when viewed in an agarose gel. This band was twice observed to yield extremely faint smaller bands when subjected to restriction digestion. The data is not shown because the bands were at the limits of visual detection in greatly overexposed photographs. Nevertheless, this result gave

us hope that we could shotgun clone the apparent DNA bands and thus amplify them.

Cloning was carried out and colonies containing recombinant plasmids were selected. However, it became apparent that the recombinant DNA was fish in origin and not viral.

DNA purification was next attempted on viral particles isolated by CsCl density gradient centrifugation. Again, extensive efforts yielded no measurable nucleic acid.

It is apparent that our efforts at DNA purification failed because of the low levels of viral particles present both in tissue and in the gradient. While particles might be visible in the electron microscope, this does not mean that there is a sufficient number to produce useful quantities of DNA. We intend to try using several pools of gradient isolated EEDV provided by Dr. McAllister but are not optimistic over the outcome. It is clear that production of the virus in tissue culture would provide a breakthrough in this area.

In an effort to circumvent the need for pure viral DNA either to produce a clone or to provide sequence information, we attempted to identify potential DNA homologies in all herpesviruses to get useful sequences. Figure one shows two regions of homology found in sequences of thymidine kinase genes of herpesviruses. The herpes simplex 1 sequence is shown in each case. Under it are listed changes in this sequence found in other herpesviruses. For each region is then shown a consensus sequence along with positions that are somewhat variable. For instance in the first region, the fifth base could be either T or C in order to match all of the

sequences. Below the consensus sequence is shown the sequence of the oligonucleotides constructed for testing. These are labeled TK1 and TK4. (The compliment of the lower region was actually used for TK4.)

These sequences are probably too variable to be used routinely as probes for herpesviruses in general and EEDV in particular. However, it was hoped that they could be used to identify similar regions in the CCV genome. The CCV sequences could be determined. It should be true that CCV sequences should be close to EEDV sequences since they are both fish herpesviruses. (The regions of conservation were primarily determined from mammalian viruses.) The determined CCV sequence could then be used to construct more specific probes for EEDV. In this way we hoped to bootstrap our way into a probe for EEDV.

We first needed to determine if TK1 and TK4 would hybridize to CCV. Figure two shows a stained agarose gel of two different CCV DNAs cut with two different restriction enzymes, EcoRI and Hae III. Figure three shows the results of hybridizing radiolabeled TK4 against a blot of this gel. Discrete bands are labeled in each lane indicating homology between TK4 and some unique target in CCV DNA. This should be part of the thymidine kinase gene. Those bands are indicated in the stained gel (Figure two) by the arrows. The band positions differ between the two different CCV DNAs due to expected migration differences found in different CCV isolates (Colyer, et al. 1986).

Figure four shows another stained gel that was probed with TK1. The resultant autoradiogram is shown in Figure five. Again,

discrete bands are observed with some differences in positions due to CCV strain variation. But again we have an indication that this region of sequence in conserved in CCV.

It is now necessary to sequence these regions of CCV to determine their exact sequence. These regions have been cloned into plasmids (P. Lai, unpublished data) and so should be relatively easily sequenced. Once sequences have been determined, new oligonucleotides, conforming more closely to the CCV sequences can be constructed. These can then be used to directly probe DNA from lake trout for EEDV. They can also be used as primers in the Polymerase Chain Reaction. In this way, it may be possible to bypass the need for pure virus.

The period of our contract has expired, but we are still intending to conduct research on this project. We are indebted to the Great Lakes Fisheries Commission for the support provided for our work and look forward to future collaborations.

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Figure Legends

Figure one

Gene sequences available from herpesvirus thymidine kinase genes. The herpes simplex 1 sequence is listed for the two conserved sequences under consideration. Any change in the sequence for the other herpesviruses is listed for that virus. A consensus sequence derived from each sequence is shown. The sequence of the oligonucleotide TK1 and the complimentary sequence of TK4 are also shown.

Figure two

Electropherogram of two different CCV isolates (506 and 543) digested with EcoRI and Hae III. Size markers are included in lane 1. Lane 2, EcoRI digested strain 506, lane 3, Hae III digested strain 506, lane 4, EcoRI digested strain 543, lane 5, Hae III digested strain 543. Arrows indicate positions of the labeled bands in Figure three.

Figure three

Autoradiogram of a blot of the gel in Figure two hybridized with TK4.

Figure four

Electropherogram of two different CCV isolates (506 and 543) digested with EcoRI and Hae III. Size markers are included in lane 1. Lane 2, EcoRI digested strain 506, lane 3, EcoRI digested strain 543, lane 4, Hae III digested strain 506, lane 5, Hae III

digested strain 543. Arrows indicate positions of the labeled bands in Figure five.

Figure five

Autoradiogram of a blot of the gel in Figure four hybridized with TK1.