Epizootic Epitheliotropic Disease of lake trout (*Salvelinus namaycush*): history and viral etiology

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ABSTRACT. A previously undescribed virus was isolated by physical (non-cultural) means from juvenile lake trout (*Salvelinus namaycush*) undergoing epizootics of unknown etiology. The enveloped virus, which measured 220 to 235 nm and had a capsid diameter of 100 to 105 nm, was extracted from moribund fish by isopycnic centrifugation and visualized by transmission electron microscopy. Virus was not found in apparently healthy fish. Horizontal transmission was demonstrated by waterborne exposure of healthy fish to 450 nm filtrates and to purified virus obtained by non-cultural methods from moribund fish, 100 % mortality occurring within 39 and 43 d post-exposure, respectively. No mortalities occurred in groups of fish exposed to 220 nm filtrates or to 450 nm filtrates prepared from healthy control fish. Investigations of natural epizootics at 3 different hatcheries revealed that fry were more affected by the disease than fingerlings. Mortalities in a population of 3.2 million fry exceeded 99.7 %. The virus appeared to be the etiologic agent of the epizootics based on the following criteria: (1) it could be obtained only from infected fish; (2) the disease could be induced with purified virus and with filtrates from infected fish prepared with filters of appropriate porosity; (3) it produced clinical signs and lesions consistent with those observed during natural epizootics; and (4) the antibiotic oxytetracycline proved ineffective against it. The virus has been tentatively classified as a herpesvirus and named Epizootic Epitheliotropic Disease Virus, based on the propensity of the agent for epithelial cells, especially those in the epidermis.

INTRODUCTION

During the past 5 yr cumulative mortalities, of unknown etiology, of hatchery-reared juvenile lake trout, *Salvelinus namaycush*, in the Great Lakes Region of the USA have exceeded 15 million. Investigations of the epizootics, which occurred at 7 hatcheries in 3 states, failed to reveal their cause (Bradley et al. 1988). McAllister & Herman (1987) and Bradley et al. (1988) observed a chlamydia-like organism associated with some of the epizootics. The disease was characterized by a rapid increase in mortalities which followed soon after the appearance of poorly pathognomonic clinical signs including swimming in a spiral pattern, congregation and gasping at the water surface, ataxia, and lethargy interspersed with periods of hyperexcitability. Smaller fish appeared to be most affected and mortalities in hatchery populations sometimes exceeded 99 %.

In this paper, we describe the extraction, purification, and partial characterization of a previously undescribed virus obtained from moribund juvenile lake trout during epizootics. A tentative virus classification and data on the pathogenicity, transmission, and etiological role of this virus are presented. The infection associated with this virus has been provisionally designated as Epizootic Epitheliotropic Disease (EED).

MATERIALS AND METHODS

Source of fish. Juvenile lake trout measuring 6 to 12 cm in length and weighing 7 to 9 g were provided by Iron River National Fish Hatchery (Iron River, WI, USA) and Marquette State Fish Hatchery (Marquette, MI, USA). Both diseased and healthy lake trout were shipped to our laboratory in insulated containers and arrived within 24 h of dispatch. In addition, epizootics

in several populations of hatchery-reared juvenile lake trout were monitored and investigated.

Extraction and purification of virus. The virus extraction procedure was carried out on diseased and on healthy, apparently uninfected fish. A 20 % tissue homogenate (weight/volume) of various organs (brain, liver, spleen, kidney, pyloric caeca, stomach, intestine, skin, and gill) was prepared in phosphate buffered saline (PBS), pH 7.2, using a sterile mortar and pestle. The homogenate was clarified by centrifugation at $10\,000 \times g$ for 30 min and again at $15\,000 \times g$ for 30 min at 4 °C. The supernatant was passed through a 450 nm membrane filter, layered onto a 3 ml 50 % CsCl cushion in a 60 ml centrifuge tube, and centrifuged at $83\,000 \times g$ for 3 h at 4 °C using a Sorvall AH-627 rotor. A visible band, present above the CsCl cushion, was harvested. Dry CsCl was added to the fraction containing the band to obtain a final density of 1.28 g cm^{-3} . Five ml of the fluid were centrifuged at $210\,000 \times g$ in a Sorvall AH-650 rotor for 18 h at 4 °C. Following centrifugation, fractions were collected from the tube and the density of each fraction deduced from its refractive index.

Electron microscopy. Samples of each fraction were stained with 1.5 % phosphotungstic acid (PTA), pH 7.0, by pseudoreplication (Smith & Melnick 1962), and examined with a Hitachi HS-9 transmission electron microscope (TEM) operating at an acceleration voltage of 75 kV.

Effect of oxytetracycline in treatment of infected lake trout. Infected fish were collected from a population undergoing an epizootic and exhibiting clinical signs of the disease. Six groups of 20 juvenile lake trout each were maintained at 10 °C in 40 l aquaria equipped with aerators and external filters. The 6 tanks of fish were randomly assigned to one of the following treatments: (1) 20 infected fish; (2) 20 infected fish and oxytetracycline (OTC); (3) 15 uninfected fish exposed to 5 infected fish; (4) 15 uninfected fish exposed to 5 infected fish and OTC; (5) 20 uninfected fish; and (6) 20 uninfected fish and OTC. The OTC was added to the tanks at a concentration of $0.05 \text{ mg ml}^{-1} \text{ d}^{-1}$. This dosage has been demonstrated to produce tissue levels in channel catfish ca 5 times that of the minimum inhibitory concentration for most bacterial pathogens of fish (Nusbaum & Shotts 1981). The OTC remained in the tanks for 5 h daily and was removed by filtration through activated charcoal. The condition of the fish and mortalities were recorded daily. Samples of moribund fish were processed for histopathology.

Infection of juvenile lake trout with EED virus. Eighty juvenile lake trout, ca 7 cm long, were divided randomly into four 40 l aquaria each containing 20 fish. Fish in the aquaria were subjected to one of the following treatments: (1) addition of 10 ml of filtrate (made

from moribund fish and obtained by low speed centrifugation followed by filtration through a 450 nm filter) to 38 l aquaria; (2) as per (1) except that the filtrate was also passed through a 220 nm filter; (3) as per (1) except that purified virus, prepared as described in extraction and purification of virus, was used; and (4) as per (1) except that filtrate from uninfected fish, prepared as in Treatment 1 (above) was used.

The tanks were contained in an environmental chamber at 10 °C and the fish were acclimated for 5 d prior to exposure. Fish were observed daily for clinical signs, morbidity, and mortality. Moribund and dead fish were processed for histopathological examination or were examined for virus using the extraction method and electron microscopy.

Species susceptibility to EED virus. The susceptibility of 5 other salmonid species (rainbow trout, *Salmo gairdneri*; brook trout, *Salvelinus fontinalis*; brown trout, *Salmo trutta*; Atlantic salmon, *Salmo salar*; and chinook salmon, *Oncorhynchus tshawytscha*) to EED virus was investigated. The rainbow, brook, and brown trout were maintained either in live cages within tanks containing infected lake trout or in troughs directly below infected lake trout. Thirty to 50 juvenile individuals of each species were exposed for 86 to 91 d to juvenile lake trout undergoing an active epizootic of EED. Water temperatures ranged from 6 to 12 °C.

Juvenile Atlantic salmon and chinook salmon were exposed to EED virus by holding them in closed 95 l aquaria with infected juvenile lake trout. Twenty individuals of each species were exposed for a period of 60 d at 10 °C. In addition, aquaria containing the same number of uninfected fish were utilized as controls.

In vitro propagation of EED virus. Propagation of EED virus was attempted in the following fish cell lines: fathead minnow (FHM) (Gravell & Malsberger 1965); rainbow trout gonad (RTG-2) (Wolf & Quimby 1962); chinook salmon embryo (CHSE-214) (Fryer et al. 1965); epithelioma papillosum cyprini (EPC) (Fijan et al. 1983); and rainbow trout hepatoma (RTH-149) (Fryer et al. 1981). Cell cultures were grown in Eagle minimal essential medium with Hank's balanced salt solution and 6 % fetal bovine serum (pH 7.0 to 7.3) at 10 or 15 °C.

Ten percent tissue homogenates were prepared from the internal organs of diseased fish and the homogenates were clarified by low speed centrifugation and by filtration through a 450 nm membrane as described for the virus extraction and purification. A 0.1 ml aliquot of the tissue fluid was inoculated onto confluent monolayers of each cell culture. The inoculated cell cultures were observed for cytopathic effects (CPE) for 21 d. The culture fluid from the first virus passage was inoculated into a second set of cell cultures and again observed for CPE.

RESULTS

Clinical signs, gross and microscopic

Affected fish exhibited a spiral swimming behavior, ataxia, and lethargy interspersed with periods of hyperexcitability. Gross external lesions included hemorrhaging at the base of the fins and in the lower quadrant of the eye; saprolegnia infections; and fin degeneration. On autopsy, the only gross lesion was a mild swelling of the kidney. The primary histopathological lesions observed were hyperplasia, hypertrophy, and necrosis of epidermal cells; edema of the secondary lamellae of the gills; renal tubule degeneration; and dilation of the glomeruli. Eosinophilic intranuclear inclusion bodies were observed in a number of epidermal cells.

Natural epizootics

EED virus was purified from moribund fish from 2 year classes at 3 hatcheries. Three of the 4 epizootics were associated with environmental or handling stress prior to increased mortalities. Epizootics occurred at temperatures from 6 to 15°C in both the spring and fall, but primarily in the spring. Fry appeared to be more susceptible to the disease than fingerlings. Mortalities in a population of 3.20 million fry reached 3.19 million individuals, ca 99.7% of the year class. In fingerlings (10 to 13 cm), however, mortalities ranged from 27 to 30.5% in populations consisting of 0.5 to 2.0 million individuals. In all cases, epizootics were preceded by severe flashing and apparent respiratory distress.

Numerous remedial measures were attempted but they had negligible effects. The measures included the addition of NaCl, formalin, benzalkonium chloride, terramycin, erythromycin, or formalin/malachite green to the hatchery water. The duration of the epizootics ranged from 2 to 3 mo. Assays of viscera from moribund fish conducted according to established procedures (McDaniel 1979) did not reveal the presence of any known fish viruses except in one epizootic in which a dual infection of EED and infectious pancreatic necrosis (IPN) viruses was found. Infections with *Pseudomonas* spp. and *Aeromonas* spp. were found in several epizootics but they were not considered to be the primary cause of mortality. In addition, branchial epitheliocystis infections were observed in fish in 3 of the 4 epizootics (Bradley et al. 1988).

Virus extraction, purification, and electron microscopy

A diffuse band with a density of 1.28 to 1.29 g cm⁻³ was collected from linear density gradients after isopycnic centrifugation of the concentrated tissue homogenate. Examination of this fraction by electron microscopy revealed the presence of numerous uniform and hexagonal particles with icosahedral symmetry (Fig. 1). Unenveloped particles measured 100 to 105 nm in diameter and appeared to have 5 capsomeres on each side of the capsid (Fig. 1). The capsomeres also appeared to have a hollow center when stained negatively with PTA (Fig. 2). Enveloped particles, 220 to 235 nm in diameter, were observed at a density of 1.25 to 1.26 g cm⁻³ (Fig. 3).

Fig. 1. Epizootic Epitheliotropic Disease Virus. Negatively stained particles isolated from moribund juvenile lake trout (*Salvelinus namaycush*) during an epizootic. Capsomeres are visible within the capsids. Bar = 50 nm

Fig. 2. EEDV Cluster of negatively stained particles showing intact and broken capsids. Free capsomeres are also present. Note hollow centers of capsomeres. Bar = 100 nm

Fig. 3. EEDV Enveloped particle isolated from moribund juvenile lake trout and stained with PTA. Bar = 50 nm

Effect of OTC on infected lake trout

In both infected and exposed fish, 0.05 mg ml^{-1} of OTC in the water had no therapeutic effect on diseased fish. In addition, the antibiotic did not prevent transmission of the disease from infected to uninfected fish (Table 1). One hundred percent mortality was observed in both infected and exposed fish with and without OTC treatment. In the infected fish, 100 % mortality was reached in 8 d, whereas 14 to 15 d were required to attain 100 % mortality in exposed fish.

Infection of juvenile lake trout with EED virus

Lake trout exposed to the 450 nm filtrate prepared from infected fish exhibited clinical signs by Day 32. The first mortality occurred 33 d post-exposure with 100 % mortality occurring by Day 39 post-exposure (Table 2). The first mortality in fish exposed to an undetermined titer of purified virus occurred at 35 d post-exposure; 100 % mortality occurred in these fish by Day 43. No mortalities were observed in fish exposed to the 220 nm filtrate prepared from infected

Table 1. *Salvelinus namaycush*. Effect of oxytetracycline (OTC) treatment on lake trout infected and exposed to Epizootic Epitheliotropic Disease Virus. Treatment consisted of 0.05 mg OTC ml⁻¹ d⁻¹. Five hours after each daily treatment, OTC was partially removed by charcoal filtration. Infected fish exhibiting clinical signs of EED were supplied by Iron River National Fish Hatchery, WI. For the exposed group 15 uninfected fish were placed in an aquarium with 5 infected fish. The first mortality of an uninfected fish occurred 10 d post-exposure with highest mortalities on Day 12 post-exposure. Day: days post-exposure to reach 100% mortality; CM: cumulative mortality (%)

Treatment	In- fected	Unin- fected	Day	CM
Infected	20	0	8	100
Infected + OTC	20	0	8	100
Exposed	5	15	15	100
Exposed + OTC	5	15	14	100
Uninfected	0	20	—	0
Uninfected + OTC	0	20	—	0

Table 2. *Salvelinus namaycush*. Experimental infection of juvenile lake trout (7.3 ± 0.8 g and 7.8 ± 0.6 cm long) with filtered tissue homogenates and purified EED virus at 10°C. Filtered homogenate: of internal organs, gills, and skin from diseased lake trout passed through a 450 nm membrane filter (450 nm), and with an additional passage through a 220 nm membrane filter (220 nm). Purified virus isolated by isopycnic centrifugation and visualized by transmission electron microscopy. The virus was derived from the same fish as the filtrates. Controls were exposed to a crude homogenate of uninfected fish filtered through a 450 nm membrane; p.i. = post-infection

Days p.i.	Filtered homogenate 450 nm	220 nm	Purified virus	Uninfected controls
32	0	0	0	0
33	2	0	0	0
34	3	0	0	0
35	4	0	1	0
36	3	0	1	0
37	5	0	0	0
38	0	0	0	0
39	3	0	4	0
40		0	5	0
41		0	2	0
42		0	1	0
43		0	6	0
Mortality (%)	100	0	100	0

fish, and control fish exposed to a 450 nm filtrate prepared from uninfected fish all survived. Virus was found in 2 six-fish pools prepared from the groups of fish exposed to the 450 nm filtrate derived from infected fish and the purified virus. No viral particles were extracted from control fish. Histological examination of moribund individuals revealed extensive hyper-

plasia and hypertrophy of epidermal epithelial cells and renal lesions similar to those observed in fish during natural epizootics. In addition, intranuclear inclusion bodies were observed in epithelial cells of the skin (Fig. 4). No significant lesions were observed in uninfected control fish sampled at the termination of the study.

Species susceptibility to EED virus

During the exposure period of 86 to 91 d, a total of 2 rainbow, 1 brook, and 1 brown trout died. Uninfected juvenile lake trout exposed to infected lake trout, utilizing the same physical design, exhibited mortalities of > 60 % in < 30 d.

Mortalities in Atlantic and chinook salmon exposed to infected lake trout were the same as those exposed to uninfected lake trout (< 5 %).

Propagation of EED virus in cell culture

No CPE was produced during a 21 d period following the inoculation of FHM, RTG-2, CHSE-214, EPC, and RTH-149 cell lines with infected tissue fluid. CPE was not observed when the tissue culture fluid was passed a second time in these cell lines.

DISCUSSION

Our studies on the etiology of EED in lake trout lead us to conclude that a virus of the herpes group is responsible for the disease.

The consistent recovery of enveloped and unenveloped 100 to 105 nm hexagonal viral particles (Figs. 1 to 3) from diseased fish and the absence of these particles in uninfected fish suggest that the viral particles were responsible for EED. Further, infectivity studies using purified virus confirmed the viral etiology of the disease in accord with River's postulates (River 1937). The purified virus was demonstrated to be capable of inducing the disease in lake trout and was re-extracted from infected moribund fish. Furthermore, the lesions found in experimentally infected fish were consistent with those observed in fish from natural epizootics. The failure of the 220 nm filtrate to produce infection may have been due to clumping of viral particles and subsequent binding to the membrane.

OTC is known to inhibit most bacteria, rickettsia, chlamydia, and mycoplasma, but not viruses. When OTC was used to treat diseased fish or prevent exposed fish from becoming infected, it was ineffective, supporting the hypothesis that the causative agent was a virus.

Fig. 4. EEDV. Intranuclear inclusion body (arrow) in the skin epithelium of juvenile lake trout infected with EED virus; $\times 1000$

Definitive classification of this virus requires further study. However, the number of capsomeres on one side of the capsid appears to be 5 (Fig. 1). This gives a total calculated number of 162 capsomeres which is typical of the family Herpesviridae (Wildy et al. 1960). In addition, the capsomeres appear to have a hollow

center (Fig. 2), another characteristic of herpesviruses. The size of the capsid (100 to 105 nm), and buoyant density of the naked particles (1.28 g cm^{-3}) all fall within the range of herpesviruses. Although numerous naked capsids were observed, enveloped viral particles were also found. All known herpesviruses have an

Table 3. Comparison of 6 fish herpesviruses with EED virus. U: unknown

Designation	Common name of virus and natural host	Size of capsid (nm)	No. capsomeres	Pathology	In vitro propagation	Source
Salmonid herpesvirus 3	Epizootic Epitheliotropic Disease Virus <i>Salvelinus namaycush</i>	100–105	162	Epidermal hyperplasia, hypertrophy, and necrosis. Edema in gills	None	This paper
Salmonid herpesvirus 1	Herpesvirus salmonis <i>Salmo gairdneri</i> <i>Oncorhynchus nerka</i>	90–95	162	Edema in liver, gill, kidney, heart, stomach, and pseudobranch. Necrosis and hemorrhage. Pancreas syncytia	RTG-2 CHSE-214	Wolf et al. (1978)
Salmonid herpesvirus 2	<i>Oncorhynchus masou</i> virus <i>Oncorhynchus masou</i> <i>Oncorhynchus kisutch</i>	110	162	Epidermal hyperplasia, liver necrosis, tumor formation	RTG-2 CHSE-214	Kimura et al. (1981a, b)
Ictalurid herpesvirus 1	Channel catfish virus <i>Ictalurus punctatus</i>	100	162	Hemorrhage, edema, necrosis in kidneys, hematopoietic tissue and other organs	BB, BF-2	Wolf & Darlington (1971) Wolf & Herman (1971)
Pleuronectid herpesvirus	Herpesvirus scophthalmus (Turbot herpesvirus) <i>Scophthalmus maximus</i>	100	U	Epidermal hyperplasia, and necrosis	None	Buchanan and Madely (1978)
Cyprinid herpesvirus (?)	Carp pox virus <i>Cyprinus carpio</i>	110	U	Chronic epidermal hyperplasia and hypertrophy	None	Schubert (1966)
Percid herpesvirus 1	Walleye epidermal hyperplasia virus <i>Stizostedion vitreum</i>	> 100	U	Epidermal hyperplasia and necrosis with syncytia formation	WC-1, WO	Kelly et al. (1983)

envelope (Wildy et al. 1960, Wolf 1973, 1982). The large number of naked capsomeres may be explained by the fragile nature of the herpesvirus envelope (Wildy et al. 1960). Exposure to CsCl and PTA during purification and staining may have caused envelope removal from many particles. The presence of intranuclear inclusion bodies (Fig. 4) in epithelial cells provides additional evidence for classification as a herpesvirus.

The similarities and differences between EEDV and 6 known fish herpesviruses are presented in Table 3. In general, EEDV is similar in size of viral capsid, number of capsomeres, presence of an envelope, and tissue tropism and pathology to most other fish herpesviruses. However, there are differences with respect to host specificity and in vitro propagation. The present study indicates that FHM, RTG-2, CHSE-214, EPC and RTH-149 cell lines do not support growth and propagation of the virus. The RTG-2 and CHSE-214 cell lines have been demonstrated to support propagation of salmonid herpesviruses Types 1 and 2 (Table 3). EEDV also appears to be very host specific and did not affect the 5 other salmonid species investigated. In addition, EEDV causes acute mortalities whereas salmonid herpesvirus Type 1 rarely causes mortalities. In contrast to salmonid herpesvirus Type 2 which causes tumor formation in survivors, no tumors were observed in lake trout chronically infected with EEDV. Based on the information provided, it appears that EEDV belongs to the family Herpesviridae. As such, we are tentatively classifying EEDV as salmonid herpesvirus Type 3. However, it is essential that an in vitro system for propagation and assay of the EED virus be established in order to fully characterize and classify the virus.

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