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Isolation of infectious pancreatic necrosis virus (serotype Ab) from diverse species of estuarine fish

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ABSTRACT: Two significant fish kills occurred in the Pamlico River estuary (North Carolina, USA), one in December 1981 and January 1982, and the other in June 1982. The first involved only the southern flounder *(Paralichthys lethostigma)*. Histopathologic examination of morbid and moribund flounder revealed extensive sloughing and necrosis of the mucosa of the pyloric caeca and intestine, and inflammation of the submucosa of the pyloric caeca. Brain and internal organ homogenates from morbid and moribund flounder were assayed on CHSE-214 cells, and a virus was isolated. Virus titers ranged from $\leq 8.4 \cdot 10^2$ to $6.3 \cdot 10^7$ $TCID_{50}$ per gram of tissue. Cross-plaque neutralization assays indicated that the southern flounder virus was infectious pancreatic necrosis virus serotype Ab. Immersion challenge showed the isolate is only slightly virulent for fry of brook trout *(Salvelinus fontinalis)*. The second fish kill involved the southern flounder and six other species: hogchoker *(Trinectes maculatus)*, Atlantic silverside *(Menidia menidia)*, spot *(Leiostomus xanthurus)*, Atlantic croaker *(Micropogon undulatus)*, silver perch *(Bairdiella chrysura)*, and striped mullet *(Mugil cephalus)*. Virus was isolated from southern founder, hogchoker, Atlantic silverside, and spot. Neutralization assays indicated that the four isolates were nearly identical; however, the diversity of species affected suggests that the virus might not have been the specific cause of mortality.

INTRODUCTION

Infectious pancreatic necrosis (IPN) is principally known as a viral disease that causes acute disease and high mortality in 1- to 4-month-old trout. Although nonsalmonids were initially regarded as refractory to infection by IPN virus, the virus has since been isolated from many freshwater species (Dorson, 1983; McAllister, 1983). Generally these isolations have been made in the absence of clinical signs of disease (Hill, 1982). Hill (1976) and Underwood et al. (1977) extended the host range of IPN-like viruses to marine mollusks and crustaceans, and more recently the virus has been isolated from marine fish. Stephens et al. (1980) and M. Newman (pers. comm.) isolated IPN virus from Atlantic menhaden *(Brevoortia tyrannus)* and blueback herring *(Alosa aestivalis)*. Hill (1982) isolated the virus from young hatchery-reared sea bass *(Dicentrarchus labrax)* and from abnormal eggs of turbot *(Scophthalmus maximus)* and Dover sole *(Solea solea)*.

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We report here the isolation of IPN virus from four species of marine fish involved in two fish kills in the Pamlico River estuary in North Carolina (USA). The first kill, in December 1981 and January 1982, involved almost exclusively southern flounder *(Paralichthys lethostigma)*; the second, in June 1982, involved at least seven species: southern flounder, hogchoker *(Trinectes maculatus)*, Atlantic silverside *(Menidia menidia)*, spot *(Leiostomus xanthurus)*, Atlantic croaker *(Micropogon undulatus)*, silver perch *(Bairdiella chrysura)*, and striped mullet *(Mugil cephalus)*. Virus was isolated from southern flounder, hogchoker, spot, and Atlantic silverside.

MATERIALS AND METHODS

Histology

Tissues from five moribund southern flounder collected during the first fish kill were sent to the Oxford (Maryland) Laboratory, National Marine Fisheries Service, for histological examination. The specimens consisted of the formalin-fixed visceral mass from each fish, and the rest of each carcass was packed on ice. On arrival at the laboratory, kidney and gill tissues were excised from carcasses. Kidney imprints were prepared and examined for the presence of *Trypanoplasma*, and the remaining kidney along with the gills were fixed in 10 % seawater-formalin. The fixed tissues were dehydrated in ethanol, cleared in xylene, and embedded in Paraplast. The embedded tissues were sectioned at 6 μm and stained with hematoxylin-eosin.

Cells, media, and viruses

Monolayer cell cultures of chinook salmon embryo (CHSE-214), rainbow trout gonad (RTG-2), bluegill fry (BF-2), brown bullhead (BB), and fathead minnow (FHM) were maintained using Eagle's minimum essential medium supplemented with 10 % fetal bovine serum (MEM-10). The CHSE-214 and RTG-2 cells were propagated at 18 °C and the BF-2, BB, and FHM cells at 25 °C.

Plaque cloned stocks of IPN virus serotypes Ab, Sp, and VR-299 and isolates from southern flounder, hogchoker, spot, and Atlantic silverside were prepared in CHSE-214 cells infected at a multiplicity of infection of 0.01–0.001 PFU/cell. The stocks were stored at −70 °C, and infectivity was determined by plaque assay as described by Wolf & Quimby (1973).

Electron microscopy

Ultrathin sections of CHSE-214 cells infected with the southern flounder virus isolate were prepared for electron microscopic examination. Monolayer cultures were infected as previously described. After 12 h incubation at 18 °C, cells were scraped from the flask, fixed in 2 % glutaraldehyde (pH 7.2), processed, and embedded in Spurr low-viscosity epoxy resin (Polysciences, Inc., Warrington, Pennsylvania), sectioned, and stained with OsO_4.

For negatively stained preparations, infected CHSE-214 cells were incubated until CPE was evident in over 90 % of the monolayer (about 72 h post-infection). The culture medium was decanted and centrifuged for 15 min at 2000 × *g* to pellet cell debris, and

the supernatant centrifuged for 60 min at 100 000 × *g*. The resultant virus concentrate was mixed with 4 % phosphotungstic acid (pH 6.0), applied to a carbon-coated grid, and examined with a Zeiss EM-5 electron microscope.

Virus purification and antiserum production

Virus was concentrated by polyethylene glycol precipitation (Macdonald & Yamamoto, 1977) and purified on linear 10–50 % sucrose gradients by centrifugation at 97 000 × *g* for 45 min at 4 °C. The virus band was removed by side puncture, dialyzed overnight against TNE buffer (0.2 M tris, 0.15 M NaCl, 0.001 M Na_2EDTA; pH 7.2), and frozen at −70 °C.

New Zealand white rabbits were injected intravenously with purified virus and both intramuscularly and subcutaneously with an emulsion of purified virus and Freund's incomplete adjuvant (1:1). The intramuscular and subcutaneous injections were repeated 2 weeks after primary inoculation. Rabbits were bled 10 to 14 days after the last injection. Sera were collected, decontaminated by membrane filtration, inactivated at 56 °C for 30 min, and stored at −70 °C.

Virus neutralization assays

Cross-plaque neutralization assays were performed between IPN virus serotypes Ab, Sp, and VR-299 and the southern flounder isolate. We also performed plaque neutralization assays using antisera to the southern flounder isolate and viruses isolated from hogchoker, spot, and Atlantic silverside. Dilutions of antiserum and virus were made in tris buffered MEM (pH 7.8) supplemented with 2 % fetal bovine serum (MEM-2 tris). Virus samples were diluted to give 80 PFU per 35-mm assay dish. Equal volumes of antiserum and virus were combined, allowed to react for 1 h at 18 °C with mixing at 15-min intervals, and assayed for residual infectivity by plaque assay. The serum dilution giving 50 % plaque reduction was calculated (Kärber, 1931), and the serological relationships between virus isolates were determined by using the titer-ratio method of Archetti & Horsfall (1959) as described by Okamoto et al. (1983).

Virus isolation

Morbid and moribund specimens were received frozen or on ice. Samples of liver, kidney, spleen, caecum, and brain were removed and assayed individually or pooled. For small fish the entire visceral mass was removed and assayed. Specimens were homogenized, diluted in 0.1 M phosphate buffered saline (pH 7.2) containing 100 IU/ml penicillin and 100 µg/ml streptomycin, and assayed on CHSE-214 cells in 96-well microculture plates (McDaniel, 1979). Plates were incubated at 18 °C and examined for 10 days for cytopathic effects (CPE).

Virus replication – cell line specificity

Drained monolayers of CHSE-214, RTG-2, BF-2, BB, and FHM cells were inoculated with the southern flounder isolate to determine the cell culture specificity. Stock virus

was diluted 10^{-1} to 10^{-9} in MEM-2 tris and 0.05 ml of each dilution inoculated into each of eight wells on a 96-well microculture plate; MEM-2 tris was used as control inoculum. After 1 h adsorption at 18 °C, 0.1 ml of MEM-10 was added to each well and incubation continued at 18 °C. Cultures were examined daily for CPE. When no further CPE developed, or 10 days had elapsed, the cell culture fluid was harvested from the highest dilution of virus inoculum showing CPE in all eight wells and titrated on CHSE-214 cells and on the original host cells.

Virulence assay in salmonid fish

To assay the southern flounder virus isolate for virulence in salmonid fish, we used the challenge protocol described by McAllister et al. (1983). Sixty-day-old (T° = 12.5 °C) brook trout *(Salvelinus fontinalis)* were placed in 1.5-l aquaria at a stocking density of 1 g fish per 25 ml water. Aquaria were supplied with 12.5 °C spring water. Fish were exposed for 5 h to 10^5 PFU/ml water of IPN virus (VR-299 serotype) or the southern flounder isolate. Control fish were exposed to PBS. Mortality was monitored for 21 days.

RESULTS

Histopathology

Superficially, the flounder examined appeared normal. No evidence of *Trypanoplasma* infection was found. Sections of the liver indicated that the parenchymal cells contained lipid or glycogen indicating that the fish had been feeding well. The heart and kidney appeared normal. Extensive sloughing of the mucosa was evident in the intestine, but some of this was probably attributable to post-mortem change. The duodenum and caeca, however, showed necrosis as well as sloughing of the mucosa (Fig. 1) and a diffuse lymphocytic infiltrate into the submucosa (Fig. 2). Because of these lesions, a diagnosis of viral enteritis was considered.

Virus isolation

When brain and internal organ homogenates from morbid and moribund southern flounder collected in December 1981 were assayed on CHSE-214 cells at 18 °C, virus was isolated from 8 of 15 fish. Virus titer ranged from $\leq 8.4 \times 10^2$ to 6.3×10^7 $TCID_{50}$/g of tissue (Table 1). In the fish kill, in June 1982, IPN virus was recovered from visceral homogenates of four species: southern flounder, hogchoker, spot, and Atlantic silverside (Table 1). Preliminary characterization of virus isolates by standard procedures indicated that they were ether-stable but sensitive to heating at 56 °C for 60 min (99.94 % inactivation; data not shown).

Electron microscopy

Electron micrographs of thin sections of CHSE-214 cells infected with the southern flounder isolate showed cytoplasmic accumulations of singly encapsidated icosahedral virus particles. Individual or small aggregates of virus were randomly distributed throughout the cytoplasm; there was no evidence of large crystalline arrays, and no virus

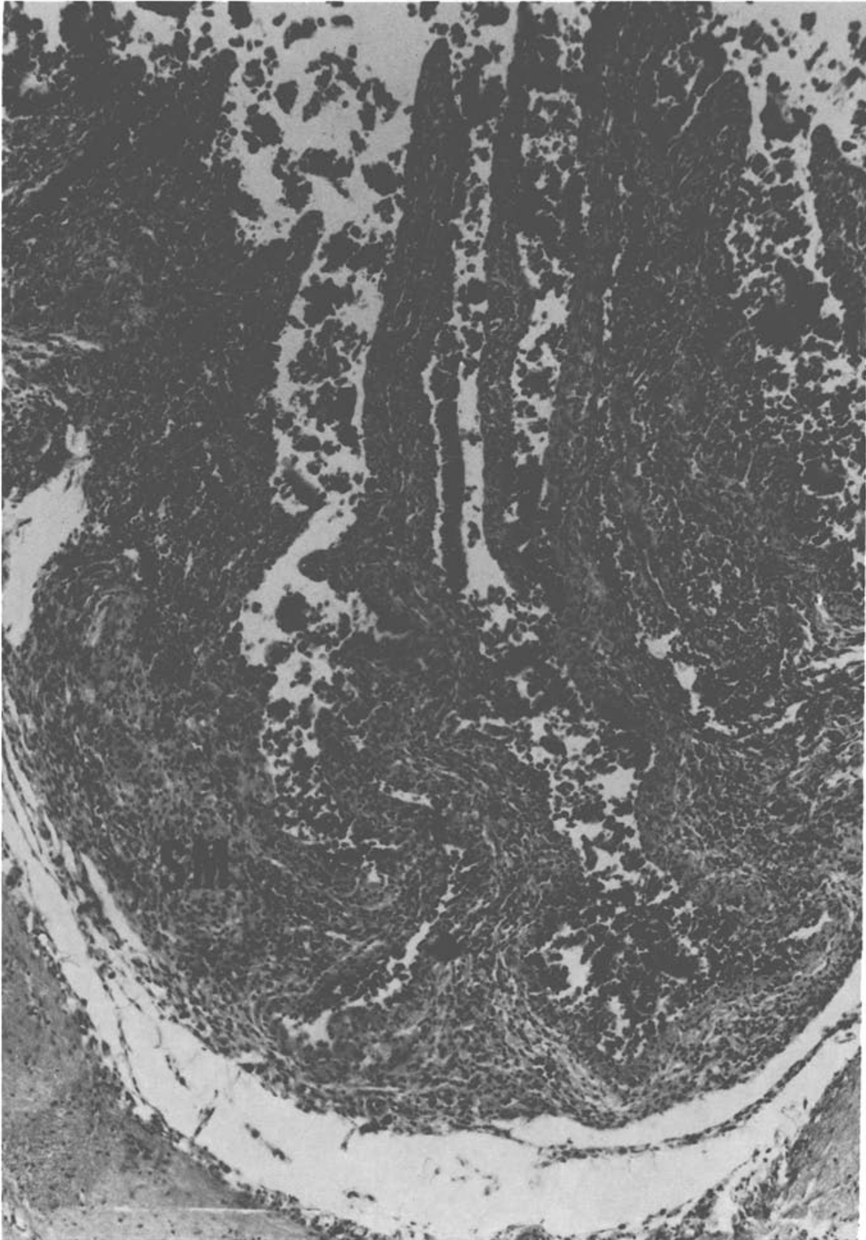

Fig. 1. Section through pyloric caeca of a moribund southern flounder showing sloughing of mucosal epithelium and inflamed submucosa (SM)

was evident in the nucleus. Negatively stained preparations of concentrated virus showed the unenveloped icosahedra to be about 57 nm in diameter (Fig. 3). The diameter of intracellular and released virus was the same.

Table 1. Quantitation of virus isolated from various tissues of marine fishes collected in Pamlico River

Species and tissue	Virus titer (range)*
Southern flounder	
Pooled liver, kidneys, spleen, and caecum	6.3×10^3– 6.3×10^7
Kidneys	1.1×10^4–$\geq 6.3 \times 10^4$
Spleen	† –$\geq 6.3 \times 10^4$
Caecum	$\leq 2.7 \times 10^3$–$\geq 6.3 \times 10^4$
Brain	† –$\leq 8.4 \times 10^2$
Hogchoker	
Viscera	$\geq 6.3 \times 10^3$
Spot	
Viscera	$\geq 6.3 \times 10^3$
Atlantic silverside	
Viscera	$\geq 6.3 \times 10^3$

* Virus titer expressed as $TCID_{50}$/g of tissue
† No viral infectivity detected

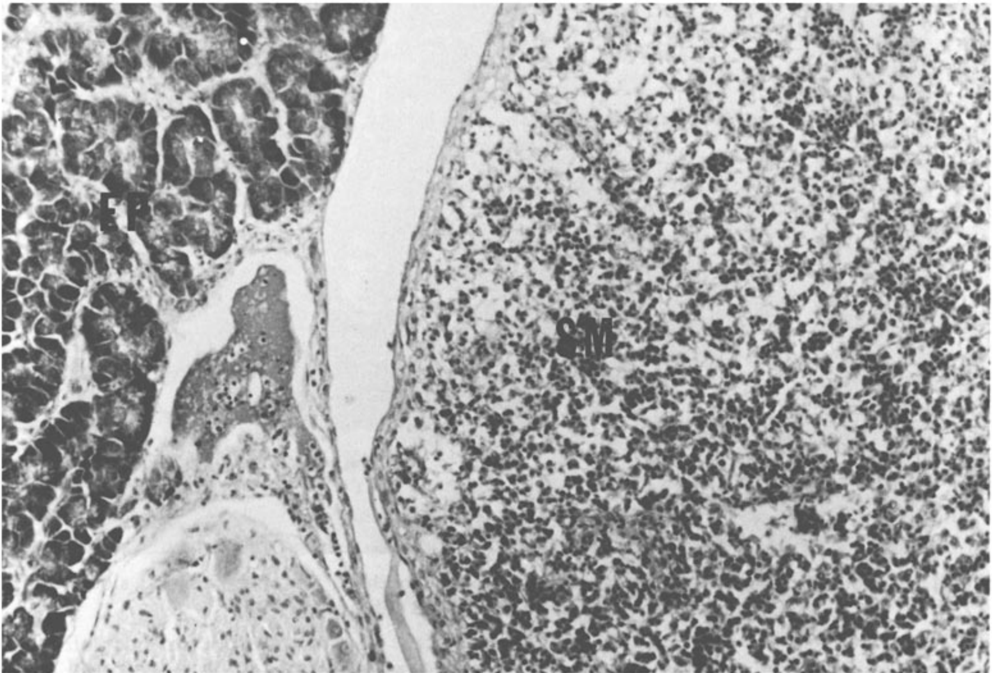

Fig. 2. Section through pyloric caeca of a moribund southern flounder showing extensive lymphocytic infiltration in the submucosa (SM) and normal appearance of exocrine pancreas (EP)

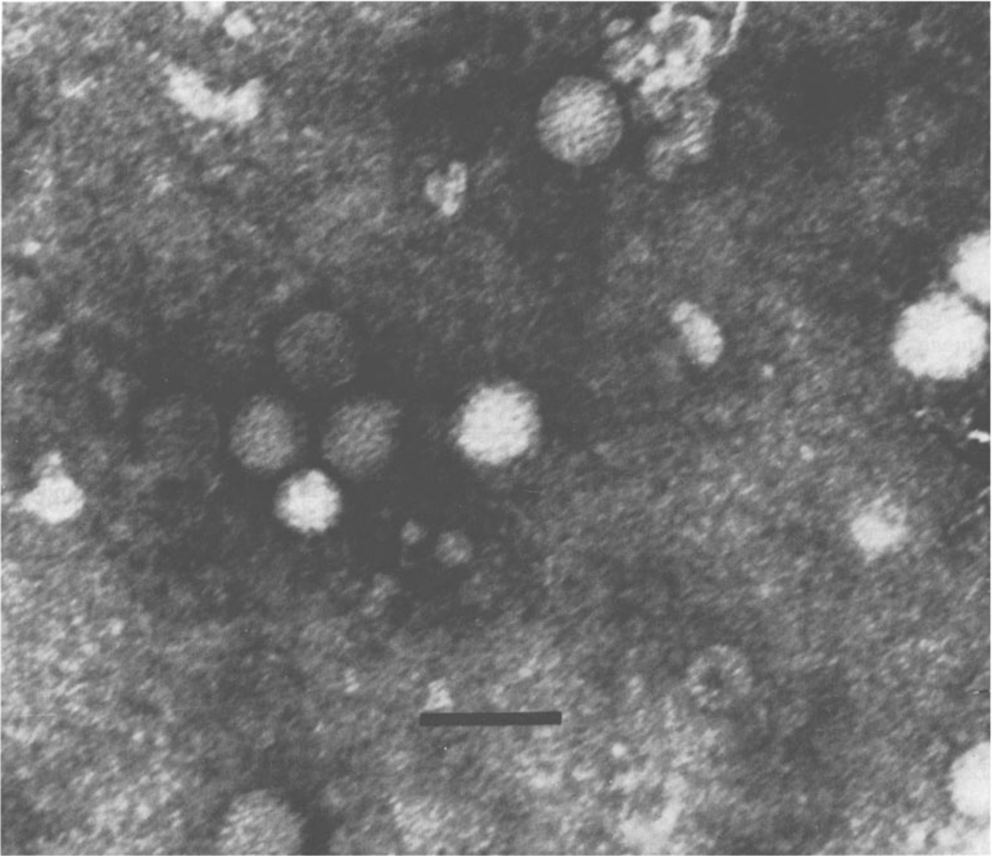

Fig. 3. Electron micrograph of negatively stained virus isolated from morbid southern flounder. Bar represents 100 nm

Cross-neutralization assays

Preliminary neutralization assays showed the southern flounder isolate was slightly neutralized (Log NI = 0.8) by a polyvalent antiserum to seven North American salmonid IPN virus isolates, suggesting a possible serological relationship to IPN virus. Cross-plaque assays indicated a high degree of cross-reactivity between the southern flounder isolate and the salmonid IPN virus serotypes Ab, Sp, and VR-299 (Table 2). Serological relationships were defined by titer-ratio analysis of the cross-neutralization data. The southern flounder isolate was found to be closely related to both serotypes Ab and Sp (Table 3).

Plaque-neutralization assays of isolates from the hogchoker, spot, and Atlantic silverside showed them to be closely related, if not identical, to that of the southern flounder (data not shown).

Table 2. Cross-plaque neutralization assays of IPN virus (serotypes Ab, Sp, and VR-299) and the southern flounder virus isolate

Virus	Antiserum*			
	IPN virus serotype			Southern flounder
	Ab	Sp	VR-229	isolate
IPN virus serotype				
Ab	4.82	4.22	3.92	4.74
Sp	3.12	5.26	4.21	4.66
VR-299	2.39	4.61	6.39	3.80
Southern flounder isolate	4.06	4.36	4.07	5.72

* Antiserum titer is expressed as the reciprocal of the logarithm of the antiserum dilution giving 50 % plaque reduction

Table 3. Serological relationship (l/r) between IPN virus (serotypes Ab, Sp, and VR-299) and the southern flounder virus isolate

Virus	Antiserum*			
	IPN virus serotype			Southern flounder
	Ab	Sp	VR-229	isolate
IPN virus serotype				
Ab	1.0	23.5	282.8	7.4
Sp		1.0	26.0	9.6
VR-299			1.0	132.5
Southern flounder isolate				1.0

* The serological relationship (l/r) between virus isolates was determined from analysis of cross-plaque neutralization assays by the titer-ratios method of Archetti & Horsfall (1950) as described by Okamoto et al. (1983)

Cell line specificity

The southern flounder IPN virus was originally isolated by using CHSE-214 cells. Four other cell lines RTG-2, BF-2, BB, and FHM were also evaluated for virus susceptibility (Table 4). The virus was passaged twice in each cell line. As judged by virus yield (2.7×10^9 $TCID_{50}$/ml) and time for manifest CPE (48–72 h), CHSE-214 cells were the most susceptible cell line. An incubation time of 120–144 h was required to manifest CPE in RTG-2 and BF-2 cells and the virus yields were lower – 6.3×10^6 and 1.5×10^7 $TCID_{50}$/ml, respectively. No CPE developed in FHM and BB cells after 10 days incubation at 18 °C.

Higher virus titers were recorded for virus harvested from RTG-2 and BF-2 cells when assayed on CHSE-214 cells, and assays of harvests from FHM and BB cells showed complete recovery of input virus. This observation further demonstrated that the CHSE-214 cell line was the most susceptible of those tested.

Table 4. Comparative response of five fish cell lines to infection with the southern flounder IPN virus isolate

Cell line	Cytopathic effect*	Virus yield ($TCID_{50}$/ml)
CHSE-214	+	2.7×10^9
RTG-2	+	6.3×10^6
BF-2	+	1.5×10^7
FHM	−	†
BB	−	†

* Cytopathic effect was characterized by rounding of the cell, pyknosis, lysis, and detachment from the flask
† No viral activity detected when virus assayed in test cell line

Susceptibility of brook trout to IPN virus isolated from southern flounder

Specific pathogen free brook trout obtained from the White Sulphur Springs (West Virginia) National Fish Hatchery were challenged by immersion with the southern flounder IPN virus isolate and with IPN virus (serotype VR-299) recovered from a natural epizootic in brook trout. Both isolates were in the second passage after primary isolation. Fish were 60 days old at challenge and were held in spring water at 12.5 °C. Exposure for 5 h to 10^5 PFU of virus/ml of water caused 89 % mortality in fish challenged with serotype VR-299 but only 5 % mortality in those exposed to the southern flounder isolate (Table 5). No control fish died. All deaths occurred 6 to 14 days after challenge; mortality was monitored for 21 days.

Table 5. Susceptibility of brook trout to the southern flounder IPN virus isolate and to IPN virus (serotype VR-299)

Virus*	Fish (died/total)	Mortality (%)
IPN virus (serotype VR-299)	33/37	89
Southern flounder isolate	2/41	5
Control	0/40	0

* Fish were challenged by immersion for 5 h in water containing 10^5 PFU/ml of virus. Control fish were exposed to PBS. Mortality was monitored for 21 days

DISCUSSION

When morbid and moribund southern flounder, collected from the Pamlico River estuary in December 1982, were examined for bacterial infection and parasites, and for toxic residues from industrial and agricultural chemicals, none of these factors could be assigned a causal role in the fish kill. Water chemistry studies and examination of nekton, phytoplankton, and benthic microinvertebrates indicated no abnormalities (Helms, 1981). However, when homogenates of brain and internal organs from flounder

were assayed on CHSE-214 cells, a virus was isolated from 8 of 15 fish. Many fish that were negative for virus were only partly intact and had been scavenged. Highest concentrations of virus ($> 10^7$ $TCID_{50}$/g tissue) were recovered from visceral organs, and virus was also recovered from brain tissue ($< 10^3$ $TCID_{50}$/g tissue). Histological examination revealed extensive sloughing and necrosis of the mucosa of the pyloric caeca and intestine and diffuse lymphocytic infiltration in the submucosa of the pyloric caeca. Kidney tissue appeared normal. Viral enteritis was considered as a contributing cause of the flounder mortality.

The southern flounder virus isolate showed characteristics similar to those of IPN virus from salmonid fish. The virus was ether-stable, inactivated by heating at 56 °C for 1 h, and had a nonenveloped, singly encapsidated, icosahedral morphology and a diameter of 57 nm. Analysis of cross-plaque neutralization assays by the titer-ratio method established that the southern flounder isolate was closely related to IPN virus serotypes Ab and Sp. The l/r value delineated by the titer-ratio analysis indicates the extent of antigenic difference or, conversely, relatedness between two viruses when both viruses and their homologous antisera are used in cross-neutralization assays. The smaller the l/r, the greater the antigenic relatedness. If $l/r = 1$, the viruses are serologically identical; values of $l/r > 20$ indicate distinct serotypes. Comparison of l/r values indicated that the southern flounder isolate was more closely related to serotype Ab ($l/r = 7.4$) than to serotype Sp ($l/r = 9.6$), and was serologically distinct from serotype VR-299 ($l/r = 132.5$). In cell culture susceptibility studies the southern flounder isolate showed a characteristic of serotype Ab in not replicating in FHM cells. The most sensitive cell line for virus detection was CHSE-214; the virus replicated in RTG-2 and BF-2 cells, but not in BB cells.

When 60-day-old brook trout, used to assay for virulence to salmonid fish, were challenged by immersion, only 5 % of the fish exposed to the southern flounder isolate died, compared with 89 % of those exposed to serotype VR-299. The southern flounder isolate was clearly avirulent. In salmonid fish, IPN virus isolates in serotype Ab are noted for avirulence, whereas those in serotype Sp are virulent. Seemingly, such consistency may not occur with IPN virus isolates from marine fish. Hill (1982), who isolated IPN virus (serotype Sp) from sea bass, found the isolate to be infectious but avirulent for rainbow trout fry. The southern flounder isolate was indeed infectious for salmonids, and the brook trout exposed to the virus became carriers. When the virus was recovered 1 year after exposure and used to challenge 42-day-old brook trout, less than 5 % of the fish died – indicating that the isolate retained its avirulence.

We also performed virulence assays with post-metamorphose summer flounder *(Paralichthys dentatus)* 3–5 cm long, and 1- to 2-year-old southern flounder. Fish were inoculated by intraperitoneal injection. Unfortunately, results were inconclusive and the southern flounder were found to be virus carriers. Virus was recovered from each of the eight control fish examined. Sera collected from southern flounder neutralized the virus isolate.

In fish from the second kill, in June 1982, virus was isolated from homogenates of visceral organs of southern flounder, hogchoker, spot, and Atlantic silverside. Plaque neutralization assays showed that virus isolates were neutralized strongly by antisera to southern flounder IPN isolate and only weakly by antisera to IPN virus (serotype VR-299). Although cross-neutralization assays are needed for confirmation, isolates from

hogchoker, spot, and Atlantic silverside appeared to be very closely related, if not identical, to southern flounder IPN virus. The diversity of species affected in the second fish kill might indicate that virus or viruses, although recovered at significant titers, might be merely adventitious agents.

Infectious pancreatic necrosis and IPN-like viruses have been isolated from an increasing diversity of fish and invertebrate species, and additional isolations will undoubtedly be made. Defining serological relationships and virulence characteristics of isolates is complex, formidable, and a continuing task. Traditionally, by virtue of antigenic character and geographical distribution, the salmonid IPN isolates are categorized into three basic serotypes: the European Ab and Sp and the North American VR-299. This rudimentary classification continues as the basic framework against which new isolates are measured – the present study included. Thus, this report of the first isolation in North America of a naturally occurring IPN virus having antigenic characteristics of IPN viruses found in Europe is of particular significance. Indeed, the IPN isolate from the southern flounder is related to both serotypes Ab and Sp. Assignment to serotype Ab was based on cell culture specificity and relative avirulence to a salmonid. It must yet be determined whether this serologic character represents an intermediate antigenic structure with bivalent reaction sites or multiple discrete antigens. Such determination may offer insight not only into the evolutionary development of IPN virus but also into alternative reagents for viral diagnostics and disease control.

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