Properties of three iridovirus-like agents associated with systemic infections of fish

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ABSTRACT: Three iridovirus-like agents associated with systemic infections in fish were examined by electron microscopy, gel electrophoresis of virion polypeptides and serological cross reactions with hyperimmune rabbit sera. The virus associated with epizootic hematopoietic necrosis (EHN) of redfin perch *Perca fluviatilis* in Australia, the virus from sheatfish *Siluris glanis* in Germany and a recently isolated iridovirus-like agent from catfish *Ictalurus melas* in France shared similar virion morphology and size, number and weight of structural polypeptides and common antigens as shown by cross immunofluorescence tests. The characteristics of these 3 piscine viruses and their striking similarity to frog virus 3, the type strain of the *Ranavirus* genus in the family Iridoviridae, suggest that they should be grouped into the same genus. The presence of similar agents in fish and amphibians emphasizes the potential adaptation and evolution of serious viral pathogens among these aquatic hosts and further complicates programs aimed at preventing or controlling the spread of fish viruses.

INTRODUCTION

The role of iridoviruses or iridovirus-like agents as causes of epidermal (lymphocystiviruses) and blood cell disorders (erythrocytic necrosis viruses) in fish is well known (Wolf 1988). Only in the past 5 yr have iridovirus-like agents been recognized as causes of serious systemic diseases among feral, cultured, and ornamental fish populations (Langdon et al. 1986, Ahne et al. 1989, Armstrong & Ferguson 1989, Pozet et al. 1992). Few properties of these systemic agents have been described but their morphological and biological characteristics clearly separate them from the piscine iridoviruses or iridovirus-like agents associated with lymphocystis, erythrocytic necrosis, cod ulcus syndrome, eel and cyprinid infections (Wolf 1988) or the recently described epidermal infections in sturgeon (Hedrick et al. 1990).

Three iridovirus-like agents causing systemic diseases in fish have been isolated using established fish cell lines. Epizootic hematopoietic necrosis (EHN) was the first serious systemic iridovirus-like infection isolated from fish (Langdon et al. 1986). Most major organs were affected but the severe necrosis in the renal and splenic hematopoietic tissues characterized the disease in a feral population of redfin perch *Perca fluviatilis* from Australia. Additional outbreaks due to the virus (EHNV) among redfin perch (Langdon & Humphrey 1987) and rainbow trout *Oncorhynchus mykiss* were reported later (Langdon & Humphrey 1987, Langdon et al. 1988). The potential host range of EHNV was shown to include at least 7 species of teleost fish (Langdon 1989). The biochemical and physical properties of the agent causing EHNV have recently been examined and confirm its affinity with the Iridoviridae (Eaton et al. 1991).

A second systemic iridovirus-like agent was isolated by Ahne et al. (1989) from sheatfish *Silurus glanis* experiencing a 100 % mortality at an intensive warm-water aquaculture facility in northwestern Germany. The serious pathogenic nature of the virus from sheatfish was demonstrated in experimental studies by Ahne et al. (1990) and Ogawa et al. (1990). Losses up to 30 % among adult market-sized sheatfish have also been attributed to the virus, which further stresses its potential economic impacts on cultured fish populations (Ahne et al. 1991).

A third iridovirus-like agent associated with systemic infections has recently been isolated from catfish *Ictalurus melas* in France (Pozet et al. 1992). A fourth systemic iridovirus-like agent with possible relationships to the 3 isolated viruses was found among dis-

eased chromide cichlids *Etropus maculatus* imported from Malaysia, but attempts to isolate the virus were unsuccessful (Armstrong & Ferguson 1989).

All 4 of these iridovirus-like agents induce similar diseases and the virus particles are of similar size and shape. The purpose of the present paper is to describe, in a side-by-side comparison, properties of the 3 agents isolated in cell culture with respect to virion size and shape, number and molecular weights of structural proteins and antigenic relationships by cross indirect fluorescent antibody tests.

MATERIALS AND METHODS

Viruses. The EHNV (13.91) used in this study was isolated from redfin perch in Australia (Langdon et al. 1986). The iridovirus-like agent (62.90) was isolated from sheatfish in Germany (Ahne et al. 1989). The third virus (59.90) was isolated from catfish in France (Pozet et al. 1992). All fish viruses were plaque-purified by 3 successive passages in the BF-2 line (CL 91; Wolf et al. 1966) from bluegill *Lepomis macrochirus* prior to use. Frozen stocks of all 4 viruses were stored at −70 to −80 °C. Frog virus 3 (FV 3) obtained directly from the American Type Culture Collection (VR-567) was grown in the *epithelioma papilloma cyprini* (EPC) line (Fijan et al. 1983).

Cell line and virus propagation. The BF-2 and EPC lines were maintained in minimal essential medium (MEM) with Earle's salts supplemented with 10 % fetal bovine serum (FBS; HyClone Lab. Inc., Logan, Utah, USA) 50 IU ml^{-1} of penicillin, 50 μg ml^{-1} of streptomycin and 2 mM L-glutamine. Cells were propagated in either 150 cm^2 flasks or 24-well dishes at 20 °C. For virus propagation, cells were infected at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU) cell^{-1}. After adsorption for 30 min at 25 °C, 20 ml of MEM with 2 % FBS was added to each of twenty 150 cm^2 flasks containing BF-2 cells which were then incubated at 20 °C until cytopathic effects (CPE) were complete (ca 4 to 6 d).

Virus purification. When CPE were complete, infected cells were frozen at −70 °C. After thawing, the suspensions were centrifuged at 5000 × *g* for 15 min to remove cellular debris. Virus in the supernatant was collected by centrifugation at 90 000 × *g* for 35 min. The virus pellet resuspended in 2.0 ml of TNE buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM disodium EDTA) was layered onto a 10 ml, 10 to 60 % (w/v) linear sucrose gradient in TNE. After centrifugation at 90 000 × *g* for 30 min, the virus bands were removed and dialyzed against 1000 volumes of TNE. Virus collected in this fashion was used immediately for negative-staining electron microscopy or for polyacrylamide gel electrophoresis.

Electron microscopy. Virus preparations were negatively stained with 1 % phosphotungstic acid (pH 6) and examined with a Philips EM400 electron microscope at 75 kV. The range, mean and standard deviation of the virion diameters were calculated by measuring 10 isolated virions.

Preparation of immune sera. Pre-immune sera was collected from 6 New Zealand white rabbits just prior to their injection with purified virus (2 rabbits with each virus). Rabbits were injected with 0.5 ml of a 1:1 mixture of purified virus and Freund's complete adjuvant into the muscle of each hind leg. After 4 wk, the rabbits received a second injection of 0.2 ml of purified virus via the marginal ear vein. The rabbits were bled and the serum collected 1 wk following the final injection. Serum was stored at 4 °C until used for neutralization and immunofluorescence assays.

Neutralization and immunofluorescence assays (IFAT). Standard serum neutralization assays of the 3 fish viruses were performed with each of the rabbit sera by plaque reduction tests. Tests were conducted with or without the addition of guinea pig complement. The cross indirect fluorescent antibody tests (IFAT) were conducted in 24-well dishes with virus-infected BF-2 cells. A 24-well plate containing monolayers of BF-2 cells was prepared for each virus. Virus was added at an MOI of 0.1 PFU cell^{-1} to all but 4 wells of the plate. After 4 d at 20 °C, when the first indications of CPE were evident, the medium was removed and the cell monolayers rinsed with 0.01 M phosphate buffered saline (PBS), pH 7.4. The cells were then fixed by the addition of 1.0 ml of a 40:60 acetone-ethanol solution at −20 °C for 10 min. The fixative was removed, and the cells were air-dried and then stored at 4 °C. Serial 2-fold dilutions in PBS (beginning with 1:10) of each sera were added so that 0.2 ml of each was added to 1 well in each of the 3 virus-infected plates. A replicate aliquot of the lowest anti-serum dilution was placed onto uninfected control cells and pre-immune sera (1:10) was added to 1 well with infected cells. After 30 min at 37 °C, the sera were removed and the wells rinsed 3 times with PBS. A 1:200 dilution of goat anti-rabbit IgG labeled with FITC (fluorescein isothiocyanate) was added to all wells. The conjugate was removed after 30 min at 37 °C by 3 rinses with PBS. After air-drying, the wells were examined for the highest dilution of serum at which immunofluorescence was detected. For photography, BF-2 and EPC cells were grown on microscope slides, infected with either the isolate 62.90 or with FV 3 at an MOI of 0.01 PFU cell^{-1}. After 3 d at 15 °C the slides were rinsed 3 times with serum-free MEM and fixed with acetone:ethanol as described above. Uninfected control and infected cells were incubated with a 1:500 dilution of anti-62.90 rabbit serum and then with the conjugate as described

for cross IFAT. After a final rinse, the slides mounted with buffered glycerol were observed with a microscope equipped for fluorescence.

Polyacrylamide gel electrophoresis. The polypeptides of the purified viruses were separated in 10 % acrylamide gels according to Laemmli et al. (1970). A panel of molecular weight markers (BioRad Inc., Richmond, California, USA) was included in the gel. After electrophoresis for 2 h at 200 V, the gels were stained with 1 % Coomassie Blue (R-250) for 1 h, destained in 40 % methanol/10 % ethanol for 1 h and then rehydrated in 10 % ethanol/5 % acetic acid. The mobility and number of viral polypeptides were calculated and their molecular weights estimated by comparison to the standards.

RESULTS

Electron microscopy

All 3 iridovirus-like agents had similar size and morphology as determined by negative-stained virus preparations. The mean diameters of the particles were consistently 153 to 154 nm (Table 1). Intact virions were dense and their icosahedral symmetry evident as demonstrated by the position of triangular vertices (Fig. 1). Larger, swollen forms that were more spherical in diameter were also observed in the 13.91 preparation (Fig. 1A). These ranged in size from 175 to 184 nm and were most likely degenerating virions.

Table 1. Comparison of virion diameters of 3 iridovirus-like agents from fish as partially purified from infected BF-2 cells and stained with 1 % phosphotungstic acid (n = 10)

| Virus | Diameter (nm) | | |
	Range	Mean	SD
13.91	148–163	153	6.4
62.90	141–163	154	8.2
59.90	141–163	153	9.0

Neutralization and immunofluorescence assays

There was no indication of neutralization in sera collected from any of the rabbits immunized with the purified viruses. Addition of complement did not show
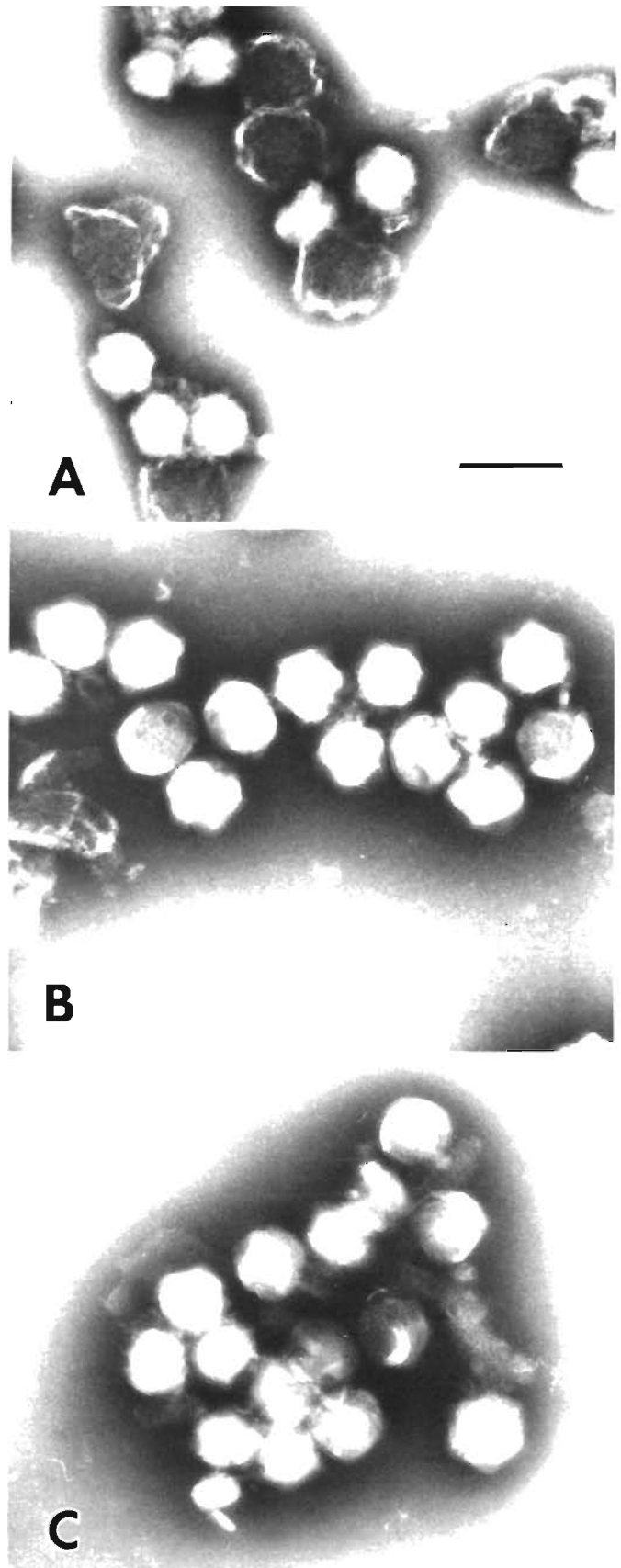

Fig. 1. Virions of 3 iridovirus-like agents isolated from fish. (A) 13.91 from redfin perch *Perca fluviatilis*, (B) 62.90 from sheatfish *Siluris glanis* and (C) 59.90 from catfish *Ictalurus melas*. Partially purified virus suspensions were stained with 1 % phosphotungstic acid. Scale bar = 200 nm
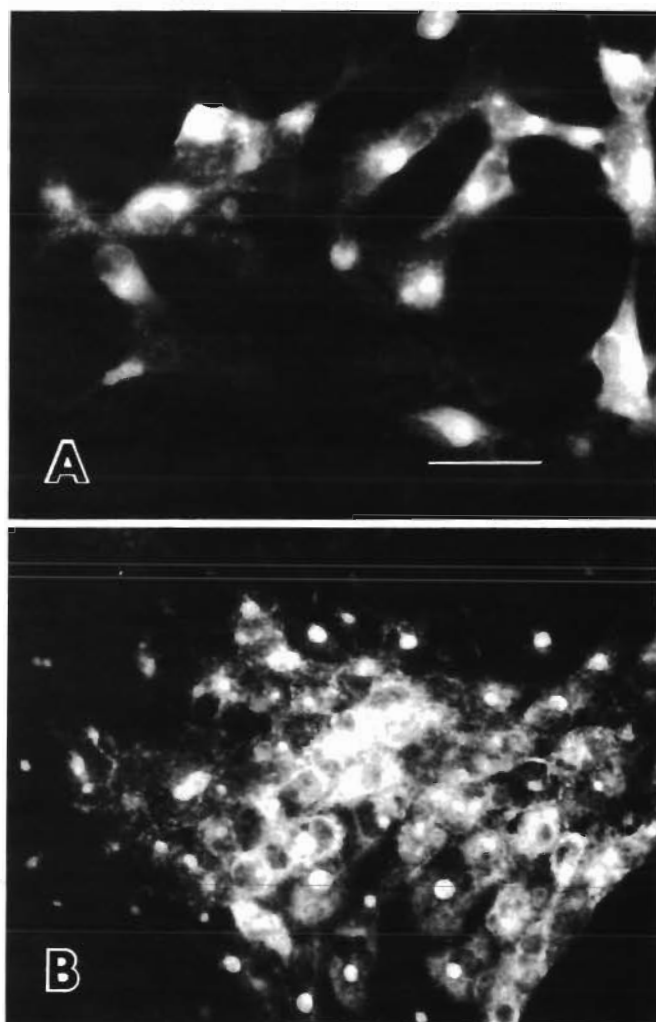
Fig. 2. Indirect immunofluorescence assays using antiserum to the iridovirus-like agent 62.90. (A) BF-2 cells infected with 62.90; (B) EPC cells infected with FV3. Scale bar = 20 μm

any effect on the neutralization. All 3 immune sera contained antibodies directed to their respective viral antigens as determined by IFAT (Table 1, Fig. 2A).

Endpoint titers of the sera against homologous virus ranged from 1600 to 12 800 (Table 2). The homologous

Table 2. Results of cross indirect immunofluorescence assays with 3 iridovirus-like agents from fish. Endpoints are expressed as the reciprocal of the highest serum dilution at which viral antigens were detected on monolayers of virus-infected BF-2 cells

Virus	Antiserum		
	13.91	62.90	59.90
13.91	12 800	3200	3200
62.90	1 600	1600	1600
59.90	1 600	3200	1600

virus and anti-serum reactions for the isolates 13.91 and 59.90 were slightly greater than with the heterologous viruses. The antisera against the isolate 62.90 reacted equally well with all 3 viruses. The staining was confined to the cytoplasm and ranged from diffuse to intense focal fluorescence of elliptical to circular bodies (Fig. 2A). There was no fluorescence detected with control serum taken prior to immunization of the rabbits.

In a separate test, antisera (1:500) against each of the 3 fish viruses were found to react equally well with their homologous isolates in BF-2 cells or with FV 3-infected EPC cells (Fig. 2B). The staining in FV 3-infected cells was similar to that observed with the 3 fish viruses with the exception that the intensely stained circular bodies were more prominent.

Polyacrylamide gel electrophoresis

The polypeptide profiles of the 3 iridovirus-like agents resembled each other and that of FV 3 (Fig. 3). Either 19 or 20 viral polypeptides were detected in each of the fish viruses compared to 15 in FV 3 (Table 3). Although the polypeptide profiles were similar for the fish and frog viruses, each virus could be distinguished by the mobility of the higher-molecular-weight polypeptides. Of the 4 viruses, the 62.90 and 59.90 strains
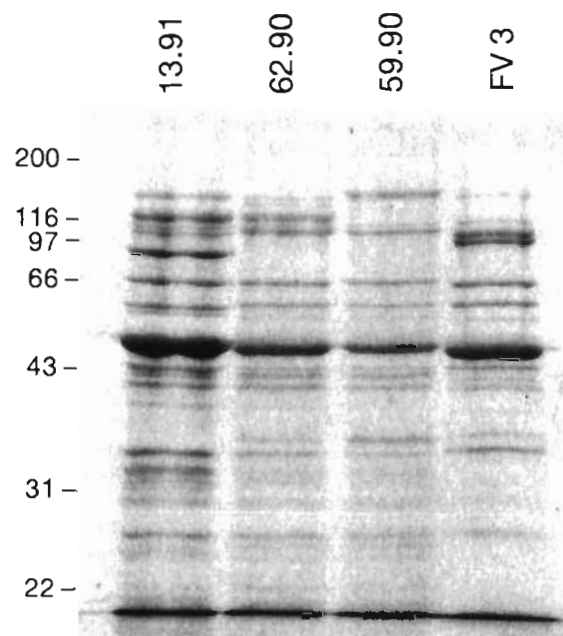

Fig. 3. Virion polypeptides of 3 iridovirus-like agents from fish (13.91, 62.90, 59.90) and frog virus 3 (FV3) as shown in a Coomassie Blue stained 10 % polyacrylamide gel. Molecular weights are in kD

Table 3. Estimated molecular weights (kD) of structural polypeptides of 3 iridovirus-like agents from fish and the amphibian iridovirus FV 3 as determined by electrophoresis in 10 % polyacrylamide gels

Virus:	13.91	62.90	59.90	FV 3
	152	152	154	152
	–	138	130	130
	–	120	–	–
	116	113	113	113
	107	110	110	–
	105	105	105	105
	85	–	–	–
	65	65	65	65
	59	59	59	59
	56	56	–	56
	48[a]	48[a]	48[a]	48[a]
	43	43	43	43
	42	–	42	42
	41	41	41	41
	39	37[a]	37[a]	37
	35[a]	36	36	36
	–	33	33	–
	32	32	32	28
	30	27	27	–
	26	26	26	25
	25	24	24	
	21	21	21	
	20			

[a] Most prominent viral polypeptides

showed the greatest number of identically sized polypeptides, with major differences found in only 5 proteins.

DISCUSSION

A comparison showed that 3 iridovirus-like agents isolated from fish suffering serious systemic diseases were related with respect to their virion morphology and size, structural proteins and antigenic properties using IFAT. We suggest that these 3 viruses are individual strains of a single group of piscine iridoviruses with strong affinities to members of the genus *Ranavirus* in the family Iridoviridae.

The family Iridoviridae contains viruses found both among invertebrate (insects) and poikilothermic vertebrate hosts (Devauchelle et al. 1985). These viruses were originally grouped together based on their icosahedral symmetry, large double-stranded DNA genomes and the presence of non-cellular-membrane-derived envelopes (Kelly & Robertson 1973). Their unique replication strategy, involving both nuclear and cytoplasmic phases (Murti et al. 1985), has led some to believe they are the missing link between the herpesviruses and poxviruses (Armnentrout & McAuslan 1974). A majority of the iridoviruses share an associa-

tion with the aquatic environment during some stage of their hosts' development and it has been proposed that their internal membrane structure confers the stability observed in aqueous solutions (Kelly 1985). Currently there are 4 genera within the family Iridoviridae (Aubertin 1991). Members of the *Iridovirus* and *Chloriridovirus* are found among insects, while viruses belonging to the *Ranavirus* and *Lymphocystivirus* are known from amphibians and fish, respectively. A group with goldfish virus, separate from the 4 genera, has also been proposed (Essani & Granoff 1989). Numerous ranaviruses have been isolated from amphibians, principally from normal adult leopard frogs *Rana pipiens* (Granoff et al. 1966). The viruses grouped in this genus show a high degree of DNA homology and are pathogenic for both tadpoles and certain toads (Essani & Granoff 1989). The few comparisons made between amphibian and piscine iridoviruses have indicated limited or no homology and this has led to their current separation into distinct genera (Essani & Granoff 1989).

The piscine and amphibian viruses share similarities with respect to broad and overlapping host cell line susceptibilities as shown for FV 3 and EHNV (Essani & Granoff 1989, Langdon 1989). The EHNV also has been shown to infect 7 species of teleost fish following natural or experimental routes of exposure (Langdon 1989). The few comparisons of amphibian and fish iridoviruses (Wolf et al. 1968, Essani & Granoff 1989) have led to the conclusion that FLDV or lymphocystis virus (Wolf 1962) and GFV, the goldfish iridovirus (Berry et al. 1983), are clearly separable from the amphibian viruses which compose a more homogeneous group. Two genera containing fish viruses in the family Iridoviridae, one including GFV and the other FLDV, were proposed, and the latter was accepted (Aubertin 1991). The results presented in our study, however, indicate that serious fish pathogens may also belong in a third genus (*Ranavirus*) previously composed solely of amphibian viruses.

The iridoviruses examined in our study were slightly larger than the 130 to 140 nm reported for members of *Ranavirus* (Essani & Granoff 1989) but their symmetry, structure and overall appearance are identical to those reported for FV 3 by Tripier-Darcy et al. (1982). Degenerating virions were commonly observed in our preparations, and these closely resemble FV 3 following pronase treatments (Tripier-Darcy et al. 1982). The virion of FV 3 is complex, with an outer envelope that surrounds a hydrated proteic capsid with an underlying membrane of lipid and protein. A majority of the virions observed in our study lacked a distinct outer envelope (Fig. 1). We presume that this was removed during purification since the chloroform sensitivity of the piscine iridoviruses suggests an important role for this external envelope (Ahne et al. 1989). Structures corres-

ponding to the external filaments described for *Lymphocystivirus* (Berthiaume et al. 1984) were not found in our study or in previous examiniations of these 3 piscine iridoviruses as observed within infected cells (Langdon et al. 1986, Ahne et al. 1989, Pozet et al. 1992).

Neutralizing antibodies have been used in attempts to compare ranaviruses. Lehane et al. (1968) compared 8 ranaviruses designated FV 1, 2 and 3, LT 1 to 4 and L-4b, but no antigenic differences were found by neutralization with hyperimmune rabbit serum. The antisera prepared by Lehane et al. (1968) against these ranaviruses was of low titer and often it is difficult to obtain any neutralizing antibodies against iridoviruses. For this reason antigenic comparisons between species are dependent on their cross reactions with non-neutralizing antibodies (Aubertin 1991). The reactivity observed by immunofluorescence with antiserum against the 3 fish iridoviruses in our study and their homologous and heterologous strains indicated the presence of conserved group-specific antigens. Additionally, cross reactions observed with antiserum to the 3 fish viruses with FV 3 further support a common grouping of the fish and amphibian viruses (Fig. 2B).

The numbers and distribution of structural proteins found in the 3 iridoviruses from fish and FV 3 were similar but all of the agents were distinct (Fig. 3, Table 3). Iridoviruses are complex viruses with large genomes, and up to 35 virus-specific polypeptides have been detected with FV 3 (Willis et al. 1977). At least 22 of these polypeptides are found in purified virions of FV 3. While 10 of the FV 3 polypeptides are phosphorylated, no evidence for glycosylated or sulfated proteins has been found (Elliot & Kelly 1980). The principal capsid polypeptide of ca 48 kD found in FV 3 (Tripier-Darcy et al. 1982) had similar major counterparts in all 3 fish iridoviruses (Fig. 3). Of the 15 polypeptides of FV 3 detected, 12 were similar in size to those detected in the fish iridoviruses. Of the 19 or 20 polypeptides found in the fish iridoviruses (isolates 59.90 and 62.90), differences were found in only 5 polypeptides, suggesting that these 2 agents are the most closely related of the 4 viruses in our study. The pattern and number of viral polypeptides observed in our study did not resemble those of FLDV or GFV, the only other piscine iridoviruses examined to date (Flugel et al. 1982, Essani & Granoff 1989). Further evidence at the genome level indicates that EHNV resembles FV 3 in being heavily methylated (Willis & Granoff 1980, Eaton et al. 1991). We presume a similar genome structure will be found with the 2 iridoviruses from catfish (62.90 and 59.90).

While future studies will be required to further define the similarities and differences between fish and amphibian iridoviruses, our study and that of Eaton et al. (1991) show that related agents exist in each of these aquatic hosts. This prompts questions regarding the

evolution and reservoirs of new viral pathogens of both fish and frogs. Secondly, it complicates fish health control programs which exclude amphibians from inspection. In the only study examining the pathogenicity of an amphibian iridovirus Wolf et al. (1968) did not find the tadpole edema virus (a ranavirus) to be pathogenic for bluegill *Lepomis macrochirus*, brook trout *Salvelinus fontinalis* or rainbow trout. Further testing of other species of fish (e.g. catfish and redfin perch) are seemingly warranted. The recent reports of systemic iridovirus infections in both fish and frogs from Europe (Ahne et al. 1989, Fijan et al. 1991) may be indicators of the potential movement or spread of these viruses between species. We can only speculate on the current geographic distribution of the systemic piscine iridoviruses but the transport of amphibians or exotic ornamental fish may be likely reasons for the appearance of similar viruses in Australia and Europe.

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