

# Immunofluorescent Study of the Replication of Infectious Pancreatic Necrosis Virus in Trout and Atlantic Salmon Cell Cultures

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Cell cultures of trout gonad tissue (RTG-2) and Atlantic salmon heart, kidney, liver, and spleen tissue were inoculated with 50 50% tissue culture infective doses (TCID<sub>50</sub>) of infectious pancreatic necrosis (IPN) virus per cell, and the titer of cell-associated and released virus was determined from 2 to 16 h postinoculation (PI). Cover slips were collected over the same period and stained for IPN viral antigen by the direct immunofluorescent (FA) technique. Viral replication was detected after a latent period of approximately 2 to 4 h and reached a peak titer of 10<sup>8.2</sup> to 10<sup>8.4</sup> TCID<sub>50</sub> per ml at 8 to 10 h PI. The release of virus was more rapid in Atlantic salmon cells than in RTG-2 cells. Viral antigen was first detected by FA from 3 to 4 h PI. Approximately 75 to 80% of the cells contained antigen in the cytoplasm 9 to 11 h PI. The direct FA technique was found to be a sensitive method for detecting IPN virus in infected cells. Three strains of IPN virus were tested for serological cross reactions by FA and virus neutralization tests.

The infectious pancreatic necrosis (IPN) virus causes a highly contagious and destructive disease of young trout (18). The IPN virus occurs in the trout-producing areas of the United States (19), Canada (3, 7), France (2, 18), and Denmark (4). A recent isolate was found in Japan (14). The virus has also been isolated from Atlantic salmon (7) and Coho salmon (16).

Although originally classified as a member of the picornavirus group (8, 15), recent studies (1, 6, 9) reported that the virus is similar in size and morphology to the reovirus and may have a double-stranded ribonucleic acid (RNA) genome. In contrast to these findings, Nicholson (10), on the basis of cytochemical and autoradiographic studies of infected cells, suggested that the genome of IPN virus is probably a single-stranded RNA. Recently, Kelly and Loh (5), on the basis of extensive electron microscopical and biochemical examination of the properties of IPN virus, concluded that IPN virus does, indeed, contain single-stranded RNA, is distinct from the reovirus and picornavirus groups, and should be placed in a new classification group.

IPN virus has been found to replicate in a

variety of fish cell cultures (15), including permanent cell lines of rainbow trout gonad (RTG-2), fathead minnow, grunt fin, and bluegill fry (BF-2) cells. Infection of RTG-2 cell cultures has been detected by the development of a cytopathic effect (CPE) (8, 10), synthesis of viral RNA (10), and electron microscopy (9). Although the virus has been isolated from Atlantic salmon (7) and Coho salmon (16), no mortalities in these fish have been attributed to IPN, and no report of the replication of IPN virus *in vitro* in Atlantic salmon cells has appeared. The present study was undertaken to determine the one-step growth curve of IPN virus in Atlantic salmon cells and to evaluate the direct immunofluorescent (FA) technique as a means of detecting IPN virus infection in trout and salmon.

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## MATERIALS AND METHODS

**Cell cultures.** The continuous rainbow trout gonad cell line, RTG-2, (17) was propagated at 20 C in growth medium (GM) consisting of Eagle minimum essential medium with Earle balanced salt solution and supplemented with 10% fetal bovine serum. Maintenance medium (MM) was the same, except for the serum concentration which was reduced to 2%. Both GM and MM contained 100 U of penicillin, 100  $\mu$ g of streptomycin, and 25 U of mycostatin per ml.

A new cell line of fibroblast-like cells was initiated in our laboratory from a trypsin digest of heart, liver, kidney, and spleen tissue of young Atlantic salmon (*Salmo salar*). This culture was subcultured 81 times over a period of 21 months and appears to be an established cell line, which we have designated AS (12). Cells of the AS line have been propagated at 20 C in GM consisting of minimal essential medium with Earle balance salt solution and supplemented with 15% fetal bovine serum.

**Viruses.** The Dry Mills strain of IPN virus was originally provided by D. Locke of the Maine Department of Inland Fisheries and Game and was propagated in RTG-2 cells in this laboratory as described previously (10, 11). This strain of IPN virus was isolated from an outbreak of IPN at the Dry Mills Hatchery, Gray, Maine, in 1967. The ATCC VR299 and Berlin, N.H., strains of IPN virus were kindly supplied by Roger Dexter, Craig Brook National Fish Hatchery, East Orland, Maine. Stock cultures of the viruses were prepared by infecting monolayer cultures of RTG-2 cells propagated at 20 C in 60-cm<sup>2</sup> culture flasks. After adsorption of the virus for 1 h at 20 C, MM was added. When CPE was extensive (usually 2 to 4 days), the remaining cells and supernatant fractions were sonically treated for 1 min at 20 kc/s with a Sonic Dismembrator (Quigley-Rochester, Inc., Rochester, N.Y.). This suspension was then clarified by centrifugation at 2,000  $\times$  g for 15 min. The virus suspensions were then filter sterilized and stored in samples at -80 C.

**Virus titration.** Viral infectivity was determined in RTG-2 cell cultures by plating 10<sup>4</sup> to 10<sup>6</sup> cells in 0.2 ml of GM into each well of Micro Test II tissue culture plates (Falcon Plastics, Oxnard, Calif.). The plates were sealed with plastic tape and incubated at 20 C until 50% confluent monolayers were obtained. The GM was then removed from each well, and 0.2 ml of each serial log dilution of the virus in MM was added to eight wells. The plates were resealed and reincubated at 20 C. After 6 days, the 50% tissue culture infective dose (TCID<sub>50</sub>) was calculated by using the procedure of Reed and Muench (13).

**Virus growth curves.** Replicate 20-cm<sup>2</sup> tissue culture flasks were seeded with RTG-2 or AS cells at a concentration of approximately 5  $\times$  10<sup>4</sup> cells per ml in the respective GM. The flasks were incubated at 20 C until the monolayers were greater than 90% confluent, approximately 2  $\times$  10<sup>6</sup> cells. After incubation, the GM was decanted, and 1 ml of stock virus (Dry Mills strain), diluted to effect an input multiplicity of 50 TCID<sub>50</sub> per cell, was added to each of the flasks. After adsorption at 20 C for 2 h, the monolayers were washed three times with MM. Each flask then received 5 ml of fresh MM and was incubated at 20 C.

At various time intervals from 1 to 20 h postinfection (PI), the medium from infected flasks was collected and centrifuged at 1,000 rpm for 15 min, and the virus titer of the supernatant fluid (released virus, RV) was determined. The cells remaining in the flasks were scraped off with a rubber policeman and collected in fresh GM. These cells were then combined with the sediment from the centrifuged medium and disrupted by sonic treatment at 20 kc/s for 1 min, and the virus titer was determined (cell-associated virus, CAV).

**Virus neutralization test.** To 1.0 ml of serial log dilutions of the immune serum was added 1.0 ml of the virus antigen, diluted to contain 1,000 TCID<sub>50</sub> per ml. After incubation at room temperature for 1 h, 0.2 ml of each virus-antiserum dilution mixture was added to four wells of a Micro Test II tissue culture plate containing semiconfluent monolayers of RTG-2 cells. After 6 days of incubation at 20 C, the plates were observed for CPE. Antiserum neutralization titers are expressed as the reciprocal of the highest dilution of the serum protecting 50% of the infected cultures, as determined by the method of Reed and Muench (13).

**Antiserum.** Antiserum to the Dry Mills strain of IPN virus was prepared in rabbits by injecting 1.0 ml of stock virus preparation intravenously at weekly intervals for 4 to 6 weeks. Sera with very high virus neutralization titers were obtained.

**FA.** Specific IPN antiserum was diluted with an equal volume of 0.15 M NaCl, and the gamma globulin fraction was precipitated twice for 30 min in the cold with half-saturated ammonium sulfate. The globulin fraction was dissolved in 0.15 M NaCl and dialyzed overnight against 0.15 M NaCl. The protein concentration was then adjusted to 1%, and the globulin was buffered with 0.05 M carbonate-bicarbonate buffer. The globulin was then dialyzed for 18 h against 10 vol of 0.05 M carbonate-bicarbonate buffer in 0.15 M NaCl containing 0.1 mg of fluorescein-isothiocyanate per ml (Baltimore Biological Laboratory, Cockeysville, Md.), followed by elution from a Sephadex G-25 column by using 0.01 M phosphate-buffered saline (PBS), pH 7.5. After overnight dialysis against 0.0175 M phosphate buffer (PB), pH 6.3, the conjugate was placed on a diethylaminoethyl-cellulose column which had been equilibrated with PB. The conjugate was then eluted with PB in 0.125 M NaCl and dialyzed overnight against 0.01 M PBS, pH 7.5. The final conjugate preparation was adsorbed on acetone-fixed monolayers of RTG-2 or AS cells just prior to use.

Cover slips were collected from Leighton tubes containing infected and uninfected cultures from 1 to 16 h PI to demonstrate IPN viral antigen by FA. The cover slips were washed three times in PBS, fixed in acetone at -20 C for 10 min, and stained by the direct fluorescent antibody technique. The cover slips were incubated with an appropriate dilution of fluorescein-conjugated anti-IPN globulin for 30 min at room temperature in a moist atmosphere. They were then washed twice in PBS, rinsed in distilled water, dried, and mounted in a mixture of PBS (10%) and glycerol (90%) at pH 8.0. The cells were examined with an AO Spencer microscope equipped for fluorescent microscopy with an Osram HBO-200 lamp, a BG-12 exciter filter, and a GG-9 barrier filter.

**RESULTS**

**Virus infectivity growth curves.** IPN virus was found to replicate in Atlantic salmon (AS) cell cultures. The titers of CAV and RV in AS cells infected at 20 C with the Dry Mills strain of IPN virus at an input multiplicity of 50 TCID<sub>50</sub> per cell are shown in Fig. 1. After a latent period of 3 to 4 h, the level of CAV increased exponentially to a maximum of 10<sup>8.2</sup> TCID<sub>50</sub> per ml at 8 h PI. The level of RV increased more slowly, reaching 10<sup>8.2</sup> TCID<sub>50</sub> per ml by 10 h PI.

The results of inoculation of RTG-2 cell cultures with the Dry Mills strain of IPN virus at the same input multiplicity were very similar, although the increase in RV appeared slower than in AS cells. CPE was observed in both infected AS and RTG-2 cultures at approximately 8 to 10 h PI. Although the appearance of infected AS and RTG-2 cells was essentially the same, the degree of CPE was usually less severe in AS cells.

**FA.** Viral antigen in RTG-2 cells infected with the Dry Mills strain of IPN virus at an input multiplicity of 50 TCID<sub>50</sub> per cell was first detected at approximately 3 h PI by FA. A small proportion of the cells (<1%) demonstrated faint cytoplasmic fluorescence. The number of fluorescent cells then increased sharply, with approximately 20% of the cells showing fairly bright, diffuse, cytoplasmic fluorescence at 4 h PI (Fig. 2). The number of cells demonstrating bright cytoplasmic fluorescence continued to

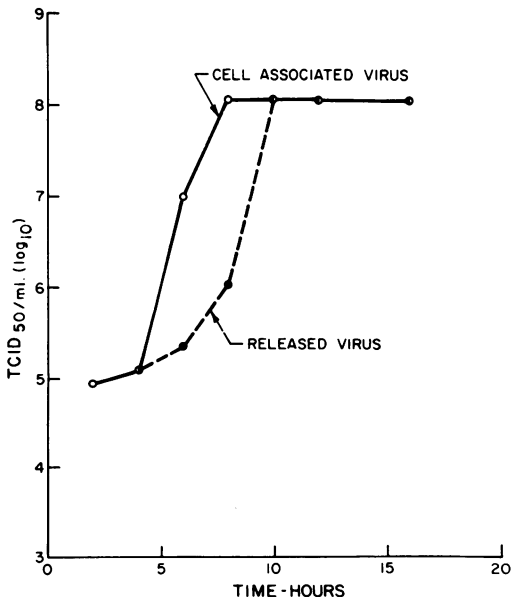


FIG. 1. Growth curves of IPN virus in AS cells at an input multiplicity of 50 TCID<sub>50</sub> per cell.

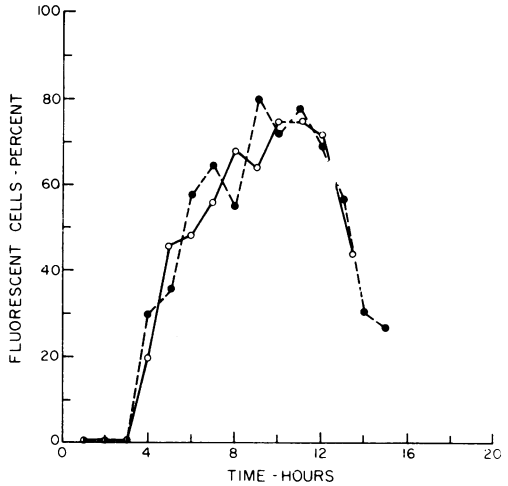
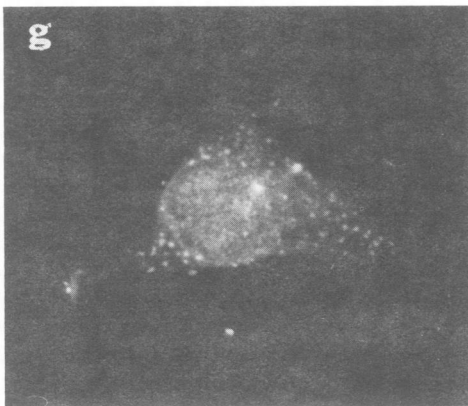
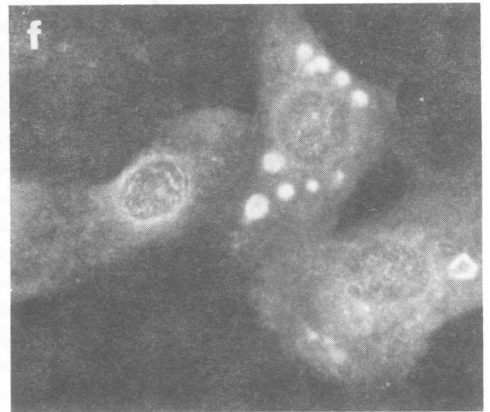
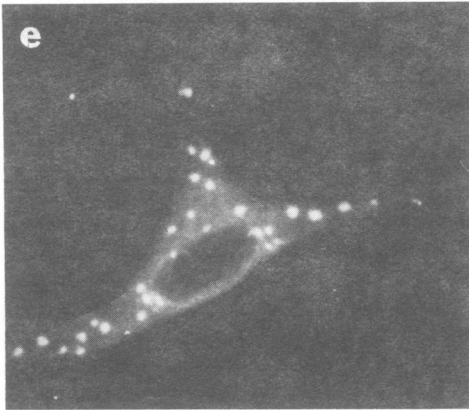
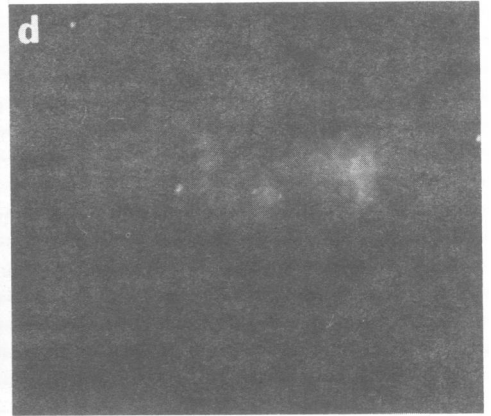
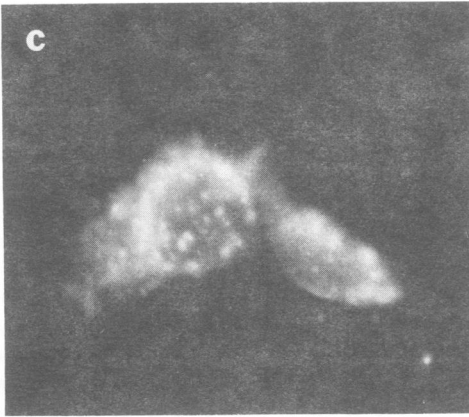
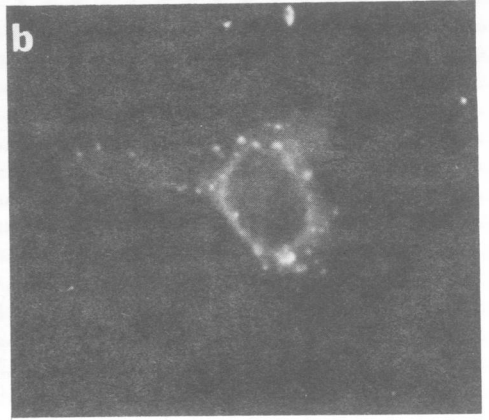
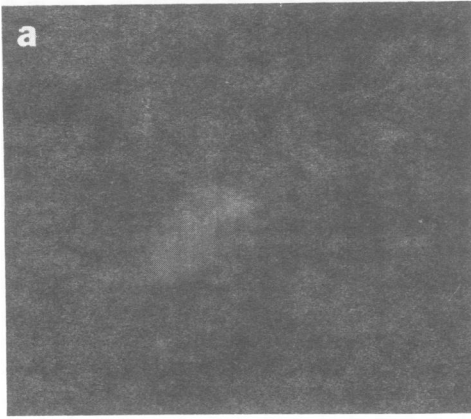


FIG. 2. IPN virus infection in RTG-2 and AS cells. Development of IPN virus antigen after infection with an input multiplicity of 50 TCID<sub>50</sub> per cell as followed with the direct fluorescent antibody (FA) technique. O, — RTG-2 cell cultures; ●, — AS cell cultures.

increase steadily until approximately 45% of the cells contained IPN antigen at 5 h PI. The rate of increase in the number of fluorescent cells slowed somewhat after 5 h PI, but continued a steady climb until 10 to 12 h PI when approximately 75% of the cells showed bright cytoplasmic fluorescence (Fig. 3a-c). As early as 5 h PI, a few cells revealed very bright fluorescent globules in addition to the more diffuse staining that filled the cytoplasm. The number of cells exhibiting these brightly stained globules increased until a late stage of infection when they were observed in most cells. The percentage of cells showing fluorescence began to decrease at 12 h PI. At this time, CPE was extensive, and many cells were sloughing off the glass.

The appearance of IPN viral antigen in AS cells infected with the Dry Mills strain of IPN virus at an input multiplicity of 50 TCID<sub>50</sub> per cell (Fig. 2) was very similar to that in RTG-2 cells. The number of cells demonstrating fluorescence increased from 1% at 3 h PI to 80% of the cell population at 9 h PI. As with infected RTG-2 cells fluorescence in infected AS cells was limited to the cytoplasm and usually appeared as fairly bright, diffuse fluorescence covering the entire cytoplasm with intensely staining globules appearing in many cells (Fig. 3d-f).

Viral antigen was first detected by FA in both RTG-2 and AS cells at 3 to 4 h PI when the virus titer had barely increased and was approximately 10<sup>4.0</sup> to 10<sup>5.0</sup> TCID<sub>50</sub> per ml. The subsequent increase in the percentage of infected cells showing cytoplasmic fluorescence closely



paralleled the increase in virus infectivity in similarly infected cells.

**Cross serological studies.** The Dry Mills strain of IPN virus was compared serologically with the ATCC VR299 and Berlin, N.H. strains by cross virus neutralization tests and cross FA staining. The results of the cross neutralization tests by using antiserum to the Dry Mill strain are shown in Table 1. The results indicate that the three strains of IPN virus are clearly antigenically distinct. The Berlin strain was inhibited by the Dry Mills immune serum to a greater extent than was the ATCC VR299 strain.

Cross FA staining was employed in an attempt to evaluate the ability of immune serum to one strain of IPN virus to stain other strains of the virus. Cover slip cultures of RTG-2 cells were infected with different strains of IPN virus at an input multiplicity of 100 TCID<sub>50</sub> per ml. At 8, 10, and 12 h PI, the cover slips were collected and stained with the anti-Dry Mills conjugate (Table 1). Cells infected with the Dry Mills strain showed brilliant specific fluorescence (rated 3+). The anti-Dry Mills conjugate stained cells infected with the Berlin, N.H., strain, demonstrated brilliant fluorescence only slightly less intense (rated 2+) than that observed with the homologous virus (Fig. 3g). The Dry Mills conjugate did not produce demonstrable fluorescence in cells infected with the ATCC VR299 strain of IPN (Fig. 3h).

Thus, the ATCC VR299 strain of IPN appears to be quite different from the Dry Mills strain. Although Dry Mills immune serum neutralized the ATCC VR299 strain to an appreciable degree, cross FA staining failed to demonstrate any relationship between the two viruses. On the other hand, the ability of a conjugate prepared with immune serum to one strain of the virus to reveal viral antigens in cells infected with a related but distinct strain of virus was demonstrated. Although cross virus neutralization tests indicated significant differences between the Dry Mills and Berlin viruses, only minor differences were evident when cross FA staining was used.

TABLE 1. Results of cross virus neutralization (VN) and fluorescent antibody (FA) tests

Virus	IPN (Dry Mills) immune serum	
	VN	FA
Dry Mills	32,500 <sup>a</sup>	3+ <sup>b</sup>
ATCC VR299	6,000	0
Berlin, N.H.	12,800	2+

<sup>a</sup> Reciprocal of the 50% neutralization end point against 1,000 TCID<sub>50</sub> of the virus.

<sup>b</sup> 0 = no fluorescence; + = slight cytoplasmic fluorescence, 2+ = moderate cytoplasmic fluorescence, 3+ = intense cytoplasmic fluorescence.

## DISCUSSION

In infectivity one-step growth curve experiments at 24 C, Moss and Gravell (9) showed an increase in CAV between 5 and 6 h PI, with an increase in RV occurring 2 to 3 h later. An exponential increase in infectious virus occurred between 5 and 12 h PI. Upon termination of the growth curve, yields of CAV and RV were approximately equal. These results agree closely with those presented in this report. Furthermore, this report demonstrates that IPN virus replicates efficiently in Atlantic salmon (AS) cells with kinetics similar to IPN replication in RTG-2 cells. Viral infectivity was detected in AS cells after a latent period of approximately 2 to 4 h and reached peak titers of 10<sup>8.2</sup> TCID<sub>50</sub> per ml from 8 to 10 h PI. This replication of IPN virus in AS cells is significant in view of the fact that very little is known about the susceptibility of Atlantic salmon to IPN.

When the FA technique was used to detect viral antigen in infected RTG-2 and AS cell cultures, initial cytoplasmic fluorescence was shown at 3 to 4 h PI. The percentage of cells demonstrating fluorescence increased over the next 6 to 7 h, until approximately 75 to 80% of the cells exhibited specific staining at 9 to 11 h PI. It is during this same period that infectious virus is being produced. The appearance of fluorescence in infected cells was limited to the

FIG. 3. Fluorescent antibody staining of RTG-2 and AS cell cultures at various times after infection with IPN virus at an input multiplicity of 50 or 100 TCID<sub>50</sub> per cell. a, Uninfected RTG-2 cells; no fluorescence. b, RTG-2 cells infected with the Dry Mills strain of IPN virus; development of cytoplasmic fluorescence (8 h PI). c, RTG-2 cells infected with the Dry Mills strain of IPN virus; development of late cytoplasmic fluorescence (11 h PI). d, Uninfected AS cells; no fluorescence. e, AS cells infected with the Dry Mills strain of IPN virus; development of cytoplasmic fluorescence (8 h PI). f, AS cells infected with the Dry Mills strain of IPN virus; development of late cytoplasmic fluorescence (12 h PI). g, RTG-2 cells infected with the Berlin, N.H., IPN virus and stained with Dry Mills IPN FA conjugate; bright cytoplasmic fluorescence (12 h PI). h, RTG-2 cells infected with the ATCC VR299 IPN virus and stained with Dry Mills IPN FA conjugate; no fluorescence (12 h PI).

cytoplasm. This observation is consistent with electron microscopy (9), cytochemical, and autoradiographic (10) studies which indicate that the replication of IPN virus occurs solely in the cytoplasm of infected cells. Occasionally, slight nuclear fluorescence was observed in a few cells late in the infectious process. However, at this time CPE was extensive, and such staining was probably the result of a complete breakdown in the integrity of the cells.

Preliminary results on cross FA staining indicate that antigenically different strains can be differentiated by the FA technique. Also, in at least one case, it appears that antigenically distinct strains of IPN virus can be detected in infected cells by using an FA conjugate prepared against only one of the strains. Much more work needs to be done by comparing the cross reactions of many different strains of IPN virus by using the FA technique.

The direct FA technique was found to be a sensitive method for detecting IPN virus in infected cells. It was possible to detect antigen approximately 4 h PI by FA, whereas CPE and high virus yields were not evident until 9 to 11 h PI. To our knowledge, FA techniques are not being utilized for the detection and identification of IPN virus. Our results suggest that the FA technique can be utilized as a research tool for studying the replication of IPN virus as well as a diagnostic technique for detecting IPN virus in infected cells.

#### ACKNOWLEDGMENTS

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#### LITERATURE CITED

- Argot, J., and R. G. Malsberger. 1972. Intracellular replication of infectious pancreatic necrosis virus. *Can. J. Microbiol.* **18**:865-867.
- Besse, P., and P. deKinkelin. 1965. Sur l'existence en France de la necrose pancreatique de la truite arc-en-ciel (*Salmo gairdneri*). *Bull. Acad. Vet. Fr.* **38**:185-190.
- Frantsi, C., and M. Savan. 1971. Infectious pancreatic necrosis virus—temperature and age factors in mortality. *J. Wildlife Dis.* **7**:249-255.
- Jorgenson, P. E. V., and F. Bregnballe. 1969. Infectious pancreatic necrosis in rainbow trout in Denmark. *Nord. Vet. Med.* **21**:142-148.
- Kelly, R. K., and P. C. Loh. 1972. Electron microscopical and biochemical characterization of infectious pancreatic necrosis virus. *J. Virol.* **10**:824-834.
- Lightner, D., and G. Post. 1969. Morphological characteristics of infectious pancreatic necrosis virus in trout pancreatic tissue. *J. Fish. Res. Board Can.* **26**:2247-2250.
- Mackelvie, R. M., and H. Artsob. 1969. Infectious pancreatic necrosis virus in young salmonids of the Canadian Maritime Provinces. *J. Fish. Res. Board Can.* **26**:3259-3262.
- Malsberger, R. G., and C. P. Cerini. 1963. Characteristics of infectious pancreatic necrosis virus. *J. Bacteriol.* **86**:1283-1287.
- Moss, L. H., and M. Gravell. 1969. Ultrastructure and sequential development of infectious pancreatic necrosis virus. *J. Virol.* **3**:52-58.
- Nicholson, B. L. 1971. Macromolecule synthesis in RTG-2 cells following infection with infectious pancreatic necrosis (IPN) virus. *J. Gen. Virol.* **13**:369-372.
- Nicholson, B. L. 1971. Effect of actinomycin D on the multiplication of the infectious pancreatic necrosis virus of trout. *Experientia* **27**:1362-1363.
- Nicholson, B. L., and C. Byrne. 1973. An established cell line from the Atlantic salmon (*Salmo salar*). *J. Fish. Res. Board Can.* **30**:913-916.
- Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent end-points. *Amer. J. Hyg.* **27**:493-497.
- Sano, T. 1971. Studies on viral diseases in Japanese fishes. I. Infectious pancreatic necrosis of rainbow trout: first isolation from epizootics in Japan. *Bull. Jap. Soc. Sci. Fish.* **37**:495-498.
- Wolf, K. 1966. The fish viruses. *Advan. Virus Res.* **12**:36-95.
- Wolf, K., and L. L. Pettijohn. 1970. Infectious pancreatic necrosis virus isolated from Coho salmon fingerlings. *Progr. Fish.-Cult.* **32**:17-18.
- Wolf, K., and M. C. Quimby. 1962. Established eurythermic line of fish cells *in vitro*. *Science* **135**:1065-1066.
- Wolf, K., and M. C. Quimby. 1971. Salmonid viruses: infectious pancreatic necrosis virus—morphology, pathology, and serology of first European isolations. *Arch. Gesamte. Virusforsch.* **34**:144-156.
- Wolf, K., S. F. Snieszko, C. E. Dunbar, and E. Pyle. 1960. Virus nature of infectious pancreatic necrosis in trout. *Proc. Soc. Exp. Biol. Med.* **104**:105-108.