APPLIED MICROBIOLOGY, Mar. 1974, p. 593-599
Vol. 27, No. 3

Immunofluorescent Cell Assay of Infectious Pancreatic Necrosis Virus

KUEN-CHING TU,[1] REX S. SPENDLOVE, AND RON W. GOEDE

Department of Bacteriology and Public Health, Utah State University, Logan, Utah 84322 and Utah Division of Wildlife Resources, Fisheries Experiment Station, Logan, Utah 84322

Received for publication 19 November 1973

An immunofluorescent cell (IFC) assay technique was developed for the quantification of infectious pancreatic necrosis (IPN) virus of salmonid fishes. Cover slip cultures of rainbow trout gonad (RTG-2) cells were infected with diluted virus preparations. After incubation to permit antigen development, the cells were stained with antiviral fluorescent antibody, and the number of fluorescing (infected) cells was counted. Optimal conditions for the IFC assay procedure are: (i) the use of RTG-2 cells cultured for at least 3 days at 20 C; (ii) 1-h absorption of IPN virus to RTG-2 cells at 20 C or alternatively, 4 h at 4 C; (iii) staining the infected cell cultures at 10 to 12 h postinfection. A linear relationship between the relative concentration of virus in the inoculum and the number of fluorescent cells in the first cycle of infection was observed. The IFC assay method is more sensitive than the plaque method for the assay of IPN virus.

Infectious pancreatic necrosis (IPN) disease was first reported by M'Gonigle (5) as an acute catarrhal enteritis in salmonid fingerlings. The causative agent, IPN virus, was isolated and cultivated in vitro in 1960 by Wolf et al. (15). At present the most commonly used method for identifying IPN virus depends on isolating the virus in monolayers of fish cells, followed by a neutralization test with anti-IPN virus serum (11, 12, 14). Fluorescent antibody studies with IPN virus have been reported (6; J. E. Argot, Dissertation Abstr. 5605B, 1969). Piper et al. (6) concluded that fluorescent antibody would be useful in studying the replication of IPN virus, and would also have diagnostic application. The purpose of this study was to develop an immunofluorescent cell (IFC) staining technique that would be of value in the assay of IPN virus and in the diagnosis of IPN virus infections.

This report is based in part on the dissertation submitted by Kuen-Ching Tu in partial fulfillment of the Doctor of Philosophy degree at the Utah State University.

MATERIALS AND METHODS

Media. The growth medium consisted of autoclavable Eagle minimum essential medium (16). Prescribed levels of $NaHCO_3$ and 1-glutamine, plus 0.5% fresh yeast extract, 10% fetal calf serum (FCS) and antibiotics were added after autoclaving. The final concentrations of antibiotics were 250 units of penicillin, 2.5 units of bacitracin, and 2.5 μg of neomycin and streptomycin per ml.

A slightly modified Tricine medium (9) was used for cover slip cultures. The medium had the same composition as the growth medium except that it contained 0.0075 M Tricine (Calbiochem, Los Angeles, Calif.) and 0.01 M $NaHCO_3$.

The first overlay medium for plaque assay consisted of the same nutrients as the cover slip culture medium plus 0.51% Agarose. The second overlay medium consisted of 0.01% filtered neutral red solution in the first overlay medium.

Cells. The RTG-2 cell line derived from rainbow trout gonads (13) was kindly provided by Roger P. Dexter, Craig Brook National Fish Hatchery, East Orland, Me. The cells were grown and maintained in growth medium at 20 C and were passed an additional 24 times in our laboratory during the course of this study. The Madin Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection.

Virus. One ATCC-VR 299 strain and five isolates of IPN virus (Kamas Brook, Loa Brook, Springville, Loa and Logan) were obtained from the Utah Division of Wildlife Resources, Fisheries Experiment Station, Logan, Utah. Canaan, Berlin, and Plymouth IPN virus isolates were provided by the Craig Brook National Fish Hatchery, East Orland, Me. Alchesay and Willow Beach IPN virus isolates were supplied by the National Fish Hatchery at Springville, Utah. All the isolates with the exception of Kamas Brook and the ATCC-VR 299 strain have been passed in RTG-2

[1] Present address: Bacteriology, Erie County Laboratories, Edward J. Meyer Memorial Hospital, Buffalo, N.Y. 14215.

cells twice; the latter two isolates have been passed six times in RTG-2 cells since they were acquired. The Kamas Brook and the ATCC-VR 299 strains of virus were plaque purified prior to use. The infectious hematopoietic necrosis virus was obtained from the National Fish Hatchery at Springville, Utah.

Stock virus. Almost confluent monolayers of RTG-2 cells grown in 16-oz prescription bottles were infected with 1:5 dilution of IPN virus stock. After 1.5 h of absorption at 20 C, 10 ml of growth medium (without serum) was added. Incubation was continued at 20 C until most of the cells came loose from the bottle. The virus was released from the cells by three cycles of freezing and thawing. The stock virus was stored at −20 C until used.

Purification of virus. The stock virus was concentrated and purified by the dextran sulfate-polyethylene glycol aqueous two phase system described by Albertsson (1, 2). A 72-g amount of infectious tissue culture fluid, 1.0 g of 20% (wt/wt) dextran sulfate (DS) 500 (Pharmacia Fine Chemicals, Uppsala, Sweden), 21.7 g of 30% (wt/wt) polyethylene glycol (PEG) 6000 (Union Carbide Corp., New York, N.Y.), and 5.3 g of NaCl were shaken until the NaCl dissolved. The above mixture (100 g) contained a final concentration of 0.2% (wt/wt) DS and 6.5% (wt/wt) of PEG 6000 in 1 M NaCl. The mixture was then transferred into a separatory funnel and allowed to stand at 4 C overnight. The bottom phase (about 1.3 ml) was collected and the DS was precipitated by adding 3 M KCl. The precipitate, the clear supernatant, and upper phase solutions were tested for virus infectivity by the plaque assay method. Another part of stock virus was processed as follows. Samples (10 ml) of infectious culture fluid were centrifuged for 1 h at 4 C at 40,000 rpm in an IEC SB 283 rotor (180,000 × *g* at the midpoint of the tube) on a gradient composed of 1-ml layers respectively of 50, 45, and 40% potassium tartrate. The band in each tube that contained virus was collected and dialyzed against saline. Cesium chloride was added directly to the virus dialysate (53 g of CsCl/100 ml of virus suspension) and then the virus-CsCl mixture was centrifuged in an IEC SB 405 rotor at 15 C at 50,000 rpm (200,000 × *g* at the midpoint of the tube) for 1.5 h. Centrifugation was continued an additional 22 h at 40,000 rpm (140,000 × *g* at the center of the tube). The purified virus was used for immunizing rabbits and for electron microscopy.

Preparation of antiserum. Part of the purified virus was mixed equally with Colab Algivant (Colab Laboratories, Inc., Chicago, Ill.) and the remaining virus was diluted with an equal amount of normal saline. Each rabbit then received 1 ml of saline-virus mixture intravenously and 0.5 ml of the Algivant virus in the hind footpad. Subsequent injections were given at weekly intervals. Rabbits were bled 1 week after the 4th injection. Additional blood collections were made 10 days after booster injections were administered. The sera were pooled and inactivated at 56 C for 30 min and adsorbed with RTG-2 cells to remove cellular antibodies.

IFC staining. The methods used in antibody labeling, staining, and IFC counting have been described previously (7, 8). The cells used in the immunofluorescence studies were grown on circular cover slips 15 mm in diameter.

Specificity test. The nonspecific staining property of conjugates was effectively reduced by absorbing twice with a normal trout tissue homogenate and once with RTG-2 cells. The homogenate was prepared by grinding fingerlings after their heads and tails were removed. After absorption none of the conjugates when diluted 1:10 showed any nonspecific fluorescence with uninfected RTG-2 cells or with smears prepared from the kidneys, spleen, or liver of normal trout. No fluorescent cells could be found when RTG-2 cells infected with infectious hematopoietic necrosis virus were stained with adsorbed conjugate. The conjugate also did not stain reovirus-infected Madin Darby canine kidney cells. As expected, specific staining was blocked by pretreatment of IPN virus-infected cultures with commercially obtained unconjugated rabbit anti-IPN virus serum. Fluorescent cells were observed in infected cover slip cultures pretreated with unlabeled immune rabbit serum when anti-rabbit serum conjugate was subsequently added.

Plaque assay. A modification of the plaque assay method described by McClain et al. (4) was used in this study. Monolayers of RTG-2 cells were grown in 60-mm plastic tissue culture petri dishes (Falcon Plastics Co., Oxnard, Calif.) which had been coated with acid soluble collagen extracted from calf skin (Andrew Zervins and David S. Yohn, personal communication). The monolayers were washed once with Tricine medium without serum, inoculated with 0.1 ml of diluted virus, and incubated at 20 C for 1.5 h. The plates were shaken by hand every 15 min during the incubation period. The cultures were then cooled at 4 C and placed on the surface of a block of ice. A 6-ml amount of agar overlay medium (38 C) was added into each plate. Incubation continued at 20 C; 2 days later 4 ml of an agar medium containing neutral red was added. Plaques were counted the next day.

Optimal adsorption time and infection time for IPN virus. For the determination of optimal adsorption time of IPN virus to cells, 0.02-ml inocula containing the same concentration of virus were added onto RTG-2 cell cover slip cultures. The infected cultures were then incubated at 4 or 20 C for various periods of time. At the end of each adsorption time, 5 ml of growth medium containing a 1:100 dilution of antiserum were added to the cultures. After 12 h of infection at 20 C, the cells were stained with fluorescent antibody, and the number of fluorescent cells in each culture was counted.

Determination of optimal postinfection time for IFC staining. Cover slip cultures of RTG-2 cells infected with IPN virus were incubated at 20 C. At 2-h intervals a set of infected cultures was removed, stained with fluorescent antibody, and the infected cells were counted by the IFC assay technique. The time at which the cover slip cultures had the most fluorescent cells was considered to be the optimal time for incubation.

Virus susceptibility and cell age. Cover slip

cultures of RTG-2 cells of different ages were infected with 0.02 ml of diluted IPN virus isolates, respectively. After incubation for 12 h at 20 C, the susceptibility of the cells to virus was checked by IF staining. The cultures that had the largest number of fluorescent cells were considered to be at the most susceptible age.

Sequential development of IPN virus antigen in RTG-2 cells. Four-day-old RTG-2 cover slip cultures were infected with 0.1 plaque-forming unit (PFU) of ATCC-VR 299 or Kamas Brook IPN virus per cell. After 1 h of adsorption, the cell sheets were washed three times with Eagle medium without serum. Then 5 ml of fresh Eagle medium containing a 1:100 dilution of IPN virus antiserum were added. At 2-h intervals, a set of cover slip cultures was stained with fluorescent antibody, and the cells were examined by fluorescence microscopy to determine the concentration and location of viral antigen.

Evaluation of the reproducibility of the IFC assay technique. Sixty cover slip cultures of 4-day-old RTG-2 cells were divided into three equal sets, and each culture was inoculated with 0.02 ml of inoculum. Cultures in the sets received inocula estimated to contain 190, 95, and 19 infectious viral particles, respectively. After 12 h of incubation at 20 C, the cultures were stained and the fluorescent cells on each cover slip were counted.

Micro-immunofluorescent plaque formation. Cover slip cultures of RTG-2 cells were infected at 20 C with a virus preparation containing less than 10 IFC units/0.02 ml. At 12-h intervals, cultures were harvested and stained with fluorescent antibody. Micro-immunofluorescent plaques as well as fluorescent cells were counted.

RESULTS

Concentration and purification of virus. Purification and a 53-fold concentration of IPN virus infectivity were achieved by the DS-PEG purification procedure (Table 1). Virtually all of the virus was found in the DS phase. Zonal rate centrifugation on a potassium tartrate gradient was found to concentrate the virus about 20-fold (from 3.2×10^7 to 6.2×10^8 IFC count units/ml). When the concentrated virus preparations were further purified by isopycnic centrifugation in CsCl and examined by electron microscopy, relatively little debris was observed.

TABLE 1. *Phase separation of IPN virus (Kamas Brook) in a dextran sulfate-polyethylene glycol mixture in 1 M NaCl*

Virus[a] polymer mixture	Top (PEG) phase	Infectivity titer (PFU/ml)		Recovery (%)
		Bottom[b] (DS) phase	Concn factor	
1.0×10^8	7.3×10^4	5.3×10^9	53	100

[a] Virus-polymer mixture, 100 g of the virus mixture contained 72 g of infectious culture fluid, 6.4% (wt/wt) polyethylene glycol (PEG) 6000, 0.2% (wt/wt) dextran sulfate (DS) 500 in 1 M NaCl.

[b] Bottom phase, after removal of DS by addition of KCl, had a volume of 2 ml.

Effect of adsorption time on the infectivity assay of IPN virus. Virus in 0.02 ml of inocula required 1 h to adsorb to cover slip cultures when incubated at 20 C (Fig. 1). When the cultures were incubated at 4 C the time required for adsorption increased to 4 h.

Effect of cell age on viral assays. The data summarized in Table 2 show that 1- or 2-day-old cells were less susceptible to IPN virus infection than older cells. Results varied with different viral strains. The infectivity titer of the Plymouth isolate was less than twofold higher when grown in older cells, whereas the infectivity titer of the Loa Brook isolate was as much as 17-fold higher. There was relatively little difference in the susceptibility of cells between the age of 3 and 7 days; for convenience, 3- to 4-day-old cells were used in this study.

Determination of optimal postinfection time for IFC staining. The fluorescent cells appeared as early as 6 h after infection, and then rapidly increased in numbers for the next 4 h. The fluorescent cell counts were the same at

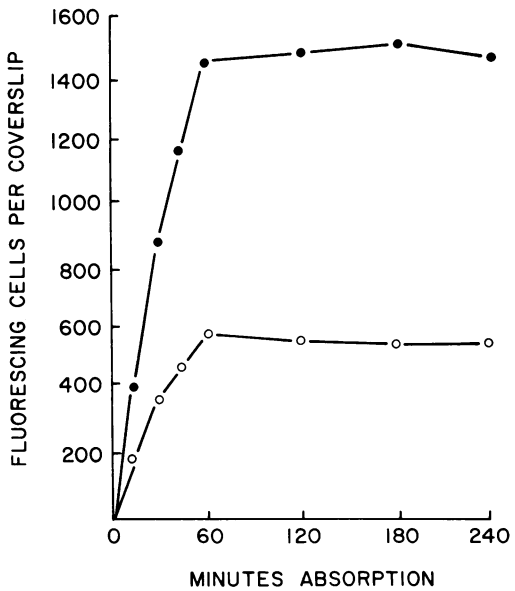

FIG. 1. *Effect of adsorption time on IPN virus infectivity in RTG-2 cells at 20 C with 0.02 ml of inoculum. The cells were grown on 15-mm circular cover slips. Symbols: ATCC-VR 299, O; Kamas Brook, ●.*

TABLE 2. *Effect of RTG-2 cell age on susceptibility to IPN virus*

IPN virus Isolate	Fold increase in infectivity titer[a]						
	1-Day-old cells	2-Day-old cells	3-Day-old cells	4-Day-old cells	5-Day-old cells	6-Day-old cells	7-Day-old cells
Kamas Brook	1.0	3.6	5.5	5.3	2.8	4.8	3.7
ATCC-VR 299	1.0	2.6	4.2	5.1	3.1	3.5	3.5
Loa Brook	1.0	8.7	13.5	16.7	9.7	10.8	10.7
Springville	1.0	5.0	7.3	11.1	8.9	7.4	6.6
Plymouth	1.0	1.3	1.5	1.7	1.4	1.9	1.7
Berlin	1.0	2.3	3.4	3.1	3.2	3.4	2.5
Canaan	1.0	2.7	2.6	3.3	2.0	2.5	2.2
Loa	1.0	1.4	2.1	2.0	2.7	1.5	2.0
Logan	1.0	2.0	3.6	3.0	3.8	3.2	3.3
Avg	1.0	3.3	4.9	5.7	4.2	4.3	4.0

[a] IFC counts obtained by using 2- to 7-day-old cells were divided by counts obtained with 1-day-old cells.

10 and 12 h postinfection; viral antigen was then released from some of the cells, and the number of fluorescent cells decreased in number. The best postinfection time for staining for IFC assay is between 10 and 12 h after infection (Fig. 2).

Sequential development of IPN virus antigen in RTG-2 cells. After 6 h of infection, very fine fluorescent granules were observed scattered throughout the cytoplasm of the cells (Fig. 3a). The granules increased in size and in number during the next 2 h (Fig. 3b). After 10 h, some large, bright fluorescent foci appeared in the cytoplasm, and at 12 h some infected cells began to release viral antigen (Fig. 3c). At 16 h postinfection, most of the infected cells had lost their viral antigen and only very weak fluorescence was observable (Fig. 3d).

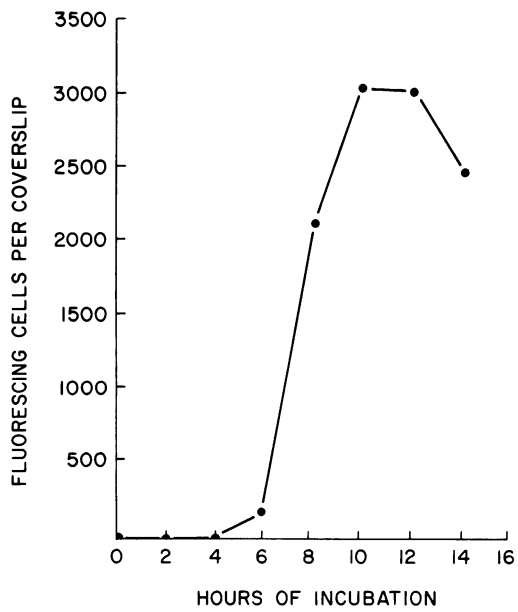

FIG. 2. *Development of IPN (ATCC-VR299) virus antigen in RTG-2 cells with inocula containing 0.01 PFU cell.*

Evaluation of the reproducibility of the IFC assay technique. The variability of the IFC assay was plus or minus 16 to 23.3% when the cultures contained 100 to 200 fluorescent cells and the whole culture or one strip across the center (approximately ⅙ of the cover slip or 30 mm^2) was counted (Table 3). There was a direct relation between the concentration of virus in the inoculum and the number of fluorescent cells in the infected cover slip cultures (Table 3). Consequently, only a single virus particle was required to produce infection in a cell.

Sensitivity of the IFC and the plaque methods. The infectivity of 16 preparations of 9 different IPN virus isolates was assayed by the IFC assay and by the plaque methods. The IFC assay results were higher by about two to six times in 14 of the 16 assays.

Micro-immunofluorescent plaque formation. The sensitivity for the detection of infectious virus was greatly increased by observing cells infected by progeny virus (Table 4). When cultures were inoculated with one or two infectious particles (as calculated from previous assays), infection could not be detected after 12 h. By 24 h of incubation, many fluorescent cells and a few microimmunofluorescent plaques began to appear, and their numbers continued to increase substantially during the subsequent 12 h. At 48 h, most of the viral antigen had been released and had spread over the entire cover slip culture.

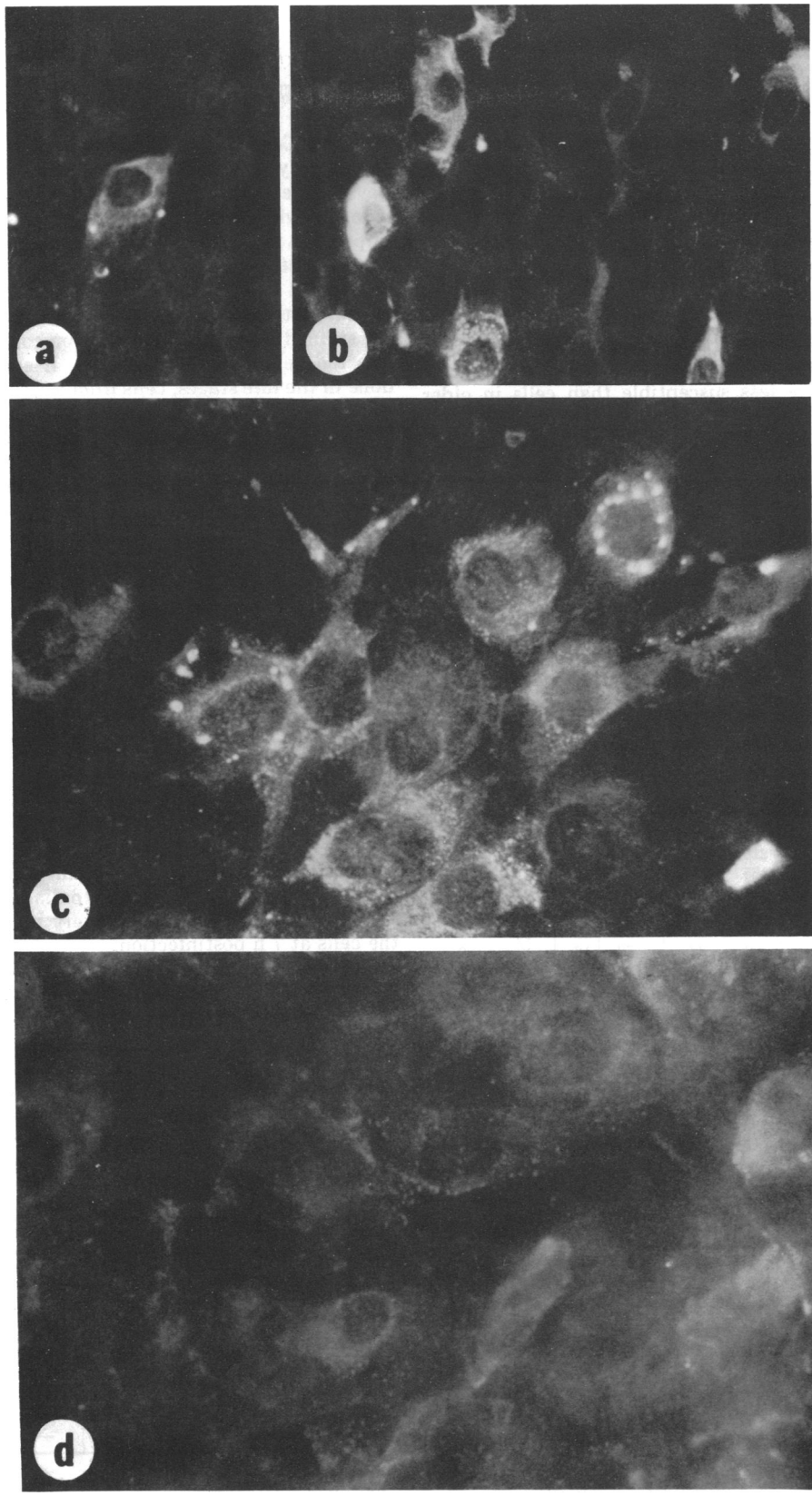

FIG. 3. *Photomicrographs of RTG-2 cells infected with IPN (ATCC-VR 299) virus per cell and stained with fluorescent antibody at various periods after infection. (a) At 6 h very fine fluorescent granules appear in the cytoplasm of some infected cells. ×750. (b) At 8 h coarse granules were observed in some cells. ×750. (c) At 12 h some cells contained coarse granules; there was evidence of antigen release from cells. ×1,400. (d) At 16 h viral antigen had been released from cells and spread over adjacent cells. Staining was weak and diffuse. ×1,400.*

DISCUSSION

The goal of this study was to develop fluorescent antibody procedures that would be useful in the quantification of IPN virus and in diagnosing IPN virus infections.

Optimal use of the anti IPN virus fluorescent antibody required that several factors affecting infection and staining be studied. The age of the RTG-2 cell cultures influenced the susceptibility of the cells. In general, cells in culture 1 or 2 days were less susceptible than cells in older cultures. Consequently, it is important to seed cells lightly and allow them to grow for several days before inoculating them with IPN virus.

In assaying viruses by the IFC assay it is important to determine the best postinfection time for staining the infected cultures. Viral antigen in cells in infected cultures does not appear at the same time in all cells. If the cells are stained too soon after infection some of the infected cells will not fluoresce. If staining is done in the late stages, cells from a second cycle of infection may be stained, or cells from the first cycle of infection will have lost antigen and will not be stained. With the IPN virus-RTG-2 cell system as described, the fluorescing cells increased in number until 10 h after infection, remained constant for 2 h, and then decreased when antigen was released from the cells. Piper et al. (6) also found that fluorescence in cells decreased beginning after 12 h of infection. Consequently, staining of IPN virus should be done between 10 and 12 h after infection.

If the procedure is to be used for the diagnosis of IPN infections then it is desirable to stain cells 6, 12, 24, 36, and 48 h after exposure to specimens. This provides for the observation of cells infected in the second cycle of infection. Table 4 shows that cultures infected with low multiplicities of infection with different strains of virus had different postinfection times at which a maximum number of stained cells were present. We were recently able to diagnose IPN virus in an outbreak in a hatchery by staining the cells at 7 h postinfection.

The precision of the IFC method for enumerating infectious IPN virus depends on the number of infected cells in a cover slip culture. Counting is accurate when the number of infected cells ranges between 30 and 2,000. The upper limit is possible since individual cells are counted and overlapping is not a problem. The feasibility of counting a single strip across the center of a cover slip culture was evaluated. The area of a cover slip of 15-mm diameter is 177

TABLE 3. *Precision of the immunofluorescent cell counting technique for assay of IPN virus*

Cover slip no.	Immunofluorescent cell count					
	WC[a]	OS	WC	OS	WC	OS
1	179	25	110	15	18	3
2	170	28	103	16	23	2
3	214	34	67	10	21	6
4	128	21	93	13	24	3
5	185	26	89	18	19	8
6	224	45	87	16	25	6
7	182	28	86	12	18	6
8	152	24	92	15	19	2
9	239	37	98	15	12	5
10	164	25	115	20	18	1
11	344	45	125	18	18	2
12	185	36	117	16	14	2
13	192	28	110	17	26	1
14	218	35	88	12	19	3
15	185	31	92	17	21	5
16	232	34	94	18	20	7
17	196	28	88	17	16	2
18	196	29	88	15	19	5
19	190	33	72	16	19	2
20	135	18	120	20	16	3
Expected IFC units in inoculum	190	31.7	95	15.9	18.3	3.7
Mean	195.5	30.5	96.7	15.8	19	3.2
S.S.D.	45.6	7.0	15.5	2.6	7.5	2.0
C.V. (%)	23.3	22.9	16	16.4	41	54

[a] WC, Whole cover slip was counted; OS, one strip across the center of cover slip was counted; S.S.D., samples' standard deviation; C.V., coefficient of variation.

TABLE 4. *Detection of IPN virus infection after inoculation of cells with a low multiplicity of infection*

Virus	Infectious virus per cover slip culture[a]	FC and IP counts							
		12 h[b]		24 h[b]		36 h[b]		48 h[b]	
		FC	IP	FC	IP	FC	IP	FC	IP
Plymouth	0.71	0	0	1	1	0	0	13	8
ATCC-VR 299	1.5	0	0	20	1	252	60	—	—
Berlin	9.2	10	0	36	46	—	—	—	—

[a] Inoculum volume was 0.02 ml. Incubation temperature was 20 C.

[b] FC, Fluorescent cells; IP, immunofluorescent plaques; 0, no fluorescent cells or immunofluorescent plaques seen; —, viral antigen spread over the cell sheet and interfered with FC and IP counting.

mm^2. The diameter of a microscopic field when a 10× eyepiece and a 10× objective are used is about 2 mm. Consequently, the area observed when one scans across the center of a cover slip is 2 by 15 mm or 30 mm^2. Thus one strip is $^{30}/_{77}$ or approximately $\frac{1}{6}$ of the total area of a cover slip. In actual counts, using the two sets of cultures having approximately 190 and 95 infected cells per cover slip, respectively (Table 3), the factor to be used with the one strip count was calculated to be 6.24. Although the one strip counts on cover slip cultures containing approximately 95 infected cells on the entire cover slip gave reproducible results, it would be advisable not to use total cover slip or single strip counts of less than 30 infected cells because of large sampling errors.

In a study of the comparison of the sensitivity between the IFC and plaque methods, Spendlove and Lennette (10) and Ibrahim and Loh (3) concluded that the IFC method is about four times more sensitive than the plaque method for the assay of vaccinia virus. The present study indicates that the IFC method is about three times more sensitive than the plaque method for the assay of IPN virus.

The results reported here show that fluorescent antibody procedures have great potential in studies requiring the assay of IPN virus and also in the diagnosis of IPN.

ACKNOWLEDGMENTS

The study was supported in part by a Public Health Service grant (AI-07750) from the National Institute of Allergy and Infectious Diseases, and by a grant from the Garcia Corporation, Englewood Cliffs, N. J. This article is Experiment Station journal paper number 1800.

LITERATURE CITED

1. Albertsson, P. A. 1960. Partition of cell particles and macromolecules. J. Wiley, New York.
2. Albertsson, P. A. 1967. Two-phase separation of virus, p. 303-321. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 2. Academy Press Inc., New York.
3. Ibrahim, A. L., and P. C. Loh. 1972. Comparison of the immunofluorescent cell counting and plaque methods for the assay of vaccinia virus. Appl. Microbiol. **23**:214-217.
4. McClain, M. E., R. S. Spendlove, and E. H. Lennette. 1967. Infectivity assay of reoviruses: comparison of immunofluorescent cell count and plaque methods. J. Immunol. **98**:1301-1308.
5. M'Gonigle, R. H. 1940. Acute catarrhal enteritis of salmonid fingerlings. Trans. Amer. Fish Soc. **70**:297-303.
6. Piper, D., B. L. Nicholson, and J. Dunn. 1973. Immunofluorescent study of the replication of infectious pancreatic necrosis virus in trout and Atlantic salmon cell cultures. Infect. Immunity **8**:249-254.
7. Spendlove, R. S. 1966. Optimal labeling of antibody with fluorescein isothiocyanate. Proc. Soc. Exp. Biol. Med. **122**:580-583.
8. Spendlove, R. S. 1967. Microscopic techniques, p. 475-520. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 3. Academy Press Inc., New York.
9. Spendlove, R. S., R. B. Crosbie, S. F. Hayes, and R. F. Keeler. 1971. Tricine-buffered tissue culture media for control of mycoplasma contaminants. Proc. Soc. Exp. Biol. Med. **137**:258-263.
10. Spendlove, R. S., and E. H. Lennette. 1962. A simplified immunofluorescent plaque method. J. Immunol. **89**:106-112.
11. Wolf, K. 1965. Infectious pancreatic necrosis: its detection and identification. Progr. Fish Cult. **27**:112.
12. Wolf, K. 1970. Guidelines for virological examination of fishes. *In* A symposium on diseases of fishes and shellfishes, p. 327-350. Special Publication no. 5, Amer. Fish. Soc., Washington, D.C.
13. Wolf, K., and M. C. Quimby. 1962. Established eurythermic line of fish cells in vitro. Science **135**:1065-1066.
14. Wolf, K., and M. C. Quimby. 1967. Infectious pancreatic necrosis (IPN): its diagnosis, identification, detection, and control. Riv. It. Piscic. Ittiopat. **11**:76-84.
15. 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.
16. Yamane, I., Y. Matsuya, and K. Jimbo. 1968. An autoclavable powdered culture medium for mammalian cells. Proc. Soc. Exp. Biol. Med. **127**:335-336.