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A VIRUS CONCENTRATION SYSTEM FOR THE  
ISOLATION OF INFECTIOUS PANCREATIC NECROSIS VIRUS  
FROM FISH HATCHERY WATER

by

SUSAN CRETER BLACKSTONE  
B.A., CARNEGIE-MELLON UNIVERSITY, 1969

A THESIS

Submitted to the University of New Hampshire  
In Partial Fulfillment of  
The Requirements for the Degree of

Doctor of Philosophy  
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Department of Microbiology  
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To my parents Edward Lewis and Florence Haskell Creter

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ABSTRACT

A VIRUS CONCENTRATION SYSTEM FOR THE ISOLATION  
OF INFECTIOUS PANCREATIC NECROSIS VIRUS FROM  
FISH HATCHERY WATER

by

SUSAN CRETER BLACKSTONE

A system for recovery of infectious pancreatic necrosis virus from water was developed making it possible to detect as few as 1 TCID unit in 100 or more gallons of surface water. The system used textile filters for collection of virus from waters sampled. Virus was collected by adsorption to viscose and fiberglass filters through which water samples were passed. Optimum virus recovery conditions included acidification of water to pH 6.0, cyclic elution of collecting filters using 5X nutrient broth, and eluate reconcentration by aqueous polymer two-phase separation. The system was used to monitor fish hatchery waters for natural infectious pancreatic necrosis virus. A total of 324 natural virus isolates were recovered during a five month test period. Virus recoveries were made before, during and after an outbreak of infectious pancreatic necrosis in hatchery fish. The isolates were

shown to be infectious pancreatic necrosis virus by serum  
neutralization tests.

## INTRODUCTION

Methods for the concentration, detection and isolation of fish viruses from large volumes (100 - 1000 l) of fish hatchery water have not been reported. Current efforts to isolate fish viruses are confined to assaying infected fish, carrier fish or fecal samples (Wolf, 1972). These methods utilize cell culture assays and are capable of isolating and identifying virus, but give no indication of the amount of virus in the hatchery water at any time. Direct isolation from the water has been possible only during outbreaks of viral disease where virus concentrations in water are great enough to permit recoveries from small test volumes (Wolf, 1966a). Consequently a need exists for a reliable method of testing large volumes of water in order to detect the presence of fish viruses. A method of this capability would permit monitoring natural or hatchery waters for virus pathogens and make it possible to take precautionary measures to protect hatchery populations.

The major objective of this research was to develop a method for the recovery of infectious pancreatic necrosis (IPN) virus from large volumes of surface waters. Analogies between the transmission of fish and human virus pathogens via water suggested elements of the technology developed for recovery of human virus pathogens might be applicable to the recovery of fish virus pathogens. Existing equipment and procedures used for the concentration of human enteric

viruses from water were tested for their application to the recovery of fish virus. Infectious pancreatic necrosis virus was used as the test fish virus pathogen in laboratory studies in which a model system was developed for the successful recovery of this virus. The usefulness of the method developed for recovery of IPN virus was tested in field trials at the National Fish Hatchery, Berlin, New Hampshire.

## LITERATURE REVIEW

Enteroviruses are potentially pathogenic to man and because a single virion may be infectious, methods of concentrating small numbers of these viruses in water or wastewater have been proposed and evaluated. Early work was limited to sample collection methods such as grab samples (Melnick et al., 1954) which were effective only if large amounts of virus were present in the water. A method for concentration of the viruses in water was required. Suspension of a gauze pad in flowing water (Melnick et al., 1954) resulted in collection of part of the virus content of surface waters. Unfortunately the method did not permit quantitative enumeration of virus since it made no provision for measuring water volume passing through the pad. A subsequent attempt to quantitate the virus recovery process was made (Liu et al., 1971), but the efficiency of the method was found to be low (Katzenelson et al., 1976). The further development of methods for the concentration of viruses in water has been based on physiochemical properties of the virus particle. In aqueous solution, the virus behaves as an amphoteric, hydrophilic colloid similar to proteins. The net charge of the virion is a function of the pH, ionic composition and ionic strength of the solution. A virus particle also possesses a measurable molecular weight and size. Using these properties, concentration methods for water borne viruses have been developed. Methods including



filter adsorption-elution, precipitation by or adsorption to polyvalent cation salts, insoluble polyelectrolytes or minerals, aqueous polymer two-phase separation, ultrafiltration, reverse osmosis, hydroextraction and electrophoresis have been reviewed (Hill et al., 1971; Fields, 1974; Sobsey, 1976). Sobsey (1976) indicates that each method for the concentration of enteroviruses has specific and unique limitations in different waters and no universal method has been developed. The best evaluated methods of virus concentration involve the use of membrane adsorption-elution techniques. In order to recover virus, a water sample must be treated in a way that removes virus from the water by adsorption to a filter. The observation that enterovirus could be made to adsorb or pass through membrane filters by changes brought about in virus suspending medium was reported by Wallis and Melnick (1967). Enhanced viral adsorption occurred following the addition of a divalent cation and lowering the pH to 5.0. It was also found that organic substances at membrane surfaces, membrane coating components (MCC), interfered with viral adsorption, and were responsible for virus passage through a filter.

The ability of a virus to reversibly adsorb to a membrane surface is in large part due to electrostatic forces. Recovery of virus from filter surfaces usually is a two step process. The first step is elution with a small volume of eluent which is usually either an alkaline proteinaceous fluid such as beef extract or nutrient broth

or an alkaline buffer of glycine (0.05-0.1 M). If the resulting eluate volume is too large to permit adequate testing for virus, a reconcentration step follows. Five virus reconcentration methods are available: 1. membrane adsorption-elution with successively smaller filters, 2. aqueous polymer two-phase separation, 3. hydroextraction 4. gel flocculation and 5. adsorption to formed precipitates. Details of these methods and their usefulness in the study are discussed under results.

Many different types of membrane filters have been used to adsorb virus. Wallis and Melnick (1967) clarified raw sewage through a fiberglass Millipore AP20 filter and a cellulose nitrate Millipore GS filter. They removed MCC with an anion exchange resin, and adsorbed virus to a cellulose ester Millipore HA filter in the presence of 0.05 M  $MgCl_2$ . Elution was accomplished with a serum eluent. Approximately 80% of the virus input was recovered. Rao and Labzoffsky (1969) utilized a fiberglass prefilter Millipore AP20 and a 0.45  $\mu m$  cellulose ester filter in the presence of 200 mg/l of  $Ca^{++}$ . Elution using 3% beef extract at pH 8.0 yielded an average recovery of 92%.

Membrane cartridge filters (Millipore Millitube, type HA porosity 0.45  $\mu m$ ) were used to recover small amounts of poliovirus type 1 from 380 l of preclarified water by Hill et al. (1972). Virus adsorption was at pH 4.5 in the presence of 1200 mg/l  $Mg^{++}$ . Elution was accomplished using 5X nutrient broth-0.05 M bicarbonate buffer pH 9.0.

Reconcentration by aqueous polymer two-phase separation was used. Virus recoveries averaged 43%.

Using fiberglass or cellulose acetate cartridge filters, Metcalf et al. (1973) concentrated poliovirus type 1 from 18 l of seeded artificial seawater with no pH adjustments and no added salt. Elution was at pH 11.5 with glycine buffer and 93% of the added virus was recovered.

Wallis et al. (1972a) have utilized a portable virus concentrator for concentration of virus in large volumes of water. The water was clarified through a series of orlon or polyester cartridge filters.  $MgCl_2$  was added to a final concentration of 0.05 M and virus was then adsorbed to a fiberglass or cellulose acetate cartridge filter. Elution with pH 11.5 glycine-NaOH buffer followed. Virus was reconcentrated by adjustment of pH to 3.5, addition of  $MgCl_2$  to a final concentration of 0.05 M and adsorption to a 90 mm diameter membrane filter. Elution of this membrane with a small volume of the same eluent resulted in 78% virus recovery and effective concentration from 1135 l to 20 ml. Improvements in this system included: use of Millipore HA membranes to clarify the water, and use of  $AlCl_3$  in a final concentration of 0.0005 M. Adsorption to cellulose ester membranes was followed by elution at pH 11.5 with a glycine-NaOH buffer and reconcentration using smaller diameter membranes and membrane adsorption-elution. This resulted in recovery of up to 94% of the added virus and an effective concentration from 1890 l to 5 ml (Wallis et al.,

1972b).

Metcalf et al. (1974) applied the use of the portable virus concentrator (Aguella-Carborundum Company, Niagara Falls, N.Y.) to polluted coastal waters. Modifications in the system were the use of fiberglass cartridge filters and fiberglass-asbestos-epoxy Cox filter membranes as adsorbing filters. Flow rates used were less than 760 ml/min. Water samples were adjusted to pH 3.5 and 0.0005M  $AlCl_3$  was used to enhance virus adsorption. Virus was recovered from filters with a glycine-NaOH eluent, pH 11.5. Reconcentration was accomplished by adsorption to and elution from successively smaller Cox filters. Recoveries of poliovirus approached 71% and an effective concentration from 95 l to 10 ml was achieved.

Sobsey et al. (1973) used the portable virus concentrator to study clean water. Fiberglass and Cox filters were used to collect virus after adjustment of sample pH to 3.5. Neither clarifying filters nor enhancing salt were used. Elution with pH 11.5 glycine-NaOH and repetitive adsorption to and elution from smaller diameter Cox filters yielded 77% virus recovery. Water volume was effectively concentrated from 378 l to 10 ml.

Hill et al. (1974) introduced the use of a Johansen proportioner (positive pressure displacement pump) for simultaneous injection of acid and adsorption-enhancing salt into water prior to passage through adsorbing filters. The proportioner was referred to as a water conditioning

apparatus. The adsorbing filters tested with its use were Millipore filters and fiberglass microfilters. Coxsackievirus B1, coxsackievirus A9, reovirus type 1 and poliovirus type 1 each were recovered from samples of 380 l. One hundred plaque forming units (PFU) of poliovirus 1 were recovered with an average recovery varying from 25 to 50%.

Jakubowski et al. (1974) described a virus-adsorbing unit of filter tubes composed of glass microfibers bonded with epoxy resin (Balston Inc.) with a porosity of 8 um. Prior to passage through the filters, the water was adjusted to pH 3.5. The virus was eluted with 0.05 M glycine buffer pH 11.5 and reconcentrated by filtering through a stacked series of epoxy fiberglass disk filters. In laboratory studies with samples consisting of 380 l, with added poliovirus, recoveries ranged from 42-57%.

In a later study, Jakubowski et al. (1975) evaluated Balston epoxy fiberglass tubes, MF nitrocellulose membranes, and AA Cox M-780 disks for their virus adsorbing efficiency. The pH was adjusted to 3.5 and the flow rate was 380 ml/min for 380 l samples. The filters were eluted with glycine-NaOH buffer, pH 11.5, and reconcentrated by membrane adsorption using Cox filters. Virus recoveries were 45.3% for the Balston filters, 30.9% for MF nitrocellulose membrane, 39% for the Cox disks and 28.8% for the K27 plus Cox disk.

The virus adsorbing effectiveness of these filters in various combinations was evaluated by Hill et al. (1976)

as part of the development of a standard method for detection of virus in drinking water. Adjustments of pH and elutions were carried out the same as in earlier studies. No significant difference in virus detection sensitivity was found with recovery efficiency ranging from 28-42%.

The flow rate of the virus concentration systems discussed was approximately 380 ml to 760 ml/min, Farrah et al. (1976) described the use of fiberglass depth cartridge filter along with a 10 in pleated epoxy fiberglass filter with flow rates of 37.8 l/min. The water was adjusted to pH 3.5 and 19,000 l were passed through the filters. Elution was accomplished using glycine buffer, pH 10.5, and the eluate reconcentrated by aluminum flocculation. Virus recoveries averaged 40-50%. Further, Farrah et al. (1977a) have reported that the pleated filters can be regenerated and reused with the same adsorptive capacity.

This system was evaluated using estuarine water (Farrah et al., 1977b) at pH 3.5 with the presence of 0.0005 M  $AlCl_3$ . Recovery of test poliovirus added to 400 l averaged 70% in 3 l of 0.05 M glycine buffer pH 11.5. Reconcentration of the eluates was made more difficult by the amount of organic compounds present. An aluminum hydroxide flocculation procedure followed by hydroextraction or ultrafiltration yielded 60-80% of the virus present in the eluate.

Fields and Metcalf (1975) concentrated adenovirus from seawater using pretreated orlon and cellulose acetate

filters to clarify the water and K27 fiberglass cartridge filters plus epoxy fiberglass Cox filters to adsorb virus. Adsorption was carried out at pH 4.5 in the presence of 0.05 M  $MgCl_2$ . Adenovirus recovery was 90%.

The aqueous polymer two-phase separation technique for concentration has a major disadvantage. The sample volume that can be handled realistically is small. It is most often used as a second step reconcentration procedure. Solubilization of two different polymers in water using certain conditions of pH, ionic strength and ionic composition will result in the development of two phases with the virus partitioned into one of these phases (Philipson et al., 1960). Virus partitioning is the result of differences in particle size and surface properties and distribution of virus in one of two liquid phases. Concentration is achieved by making the virus-containing liquid phase small, compared to its original volume. The polymers most often used are dextran sulfate (DS) and polyethylene glycol (PEG). Sodium chloride is added. Dextran sulfate can be detrimental to enterovirus and is often precipitated out with KCl, or dextran is substituted (Grindrod and Cliver, 1969).

Shuval et al. (1969) studied a one-step 173-fold concentration and a two-step 520-fold concentration method. The one-step concentration resulted in 87% recovery while the two-step resulted in 100% recovery.

Nupen (1970) examined the recovery of enterovirus

from wastewater and averaged 44% recovery with a one step process and 22% recovery with a two step procedure. Hill et al. (1972) examined the recovery of enterovirus from membrane cartridge filters using low levels of poliovirus type 1. Recoveries averaged 43% from estuarine and tap water.

Fields and Metcalf (1975) successfully employed an aqueous polymer two phase procedure to reconcentrate adenovirus from eluates of membrane cartridge filters. Virus recoveries averaged 48%.

Fattal et al. (1974) concentrated small amounts of enterovirus 140 times from 5 l volumes of tap water using the phase separation method of Shuval et al. (1969). Recoveries of poliovirus type 1 and echovirus type 7 averaged 100% and 125% respectively. Tapwater contaminated with sewage yielded only 5% recovery of natural enterovirus.

Flocculation of beef extract at low pH has been studied as a second step reconcentration process by Katzenelson et al. (1976). Comparison of pH 3.5, 4.0 and 4.5 followed by resuspension of the floc in  $\text{Na}_2\text{HPO}_4$  (0.15 M) showed that at pH 3.5 recoveries ranged from 69 to 123%, at pH 4.0 from 77 to 114%, and at pH 4.5 from 54 to 89%. The method was further evaluated using a "standard" method for isolation of virus in drinking water (a membrane adsorption technique) (Hill et al., 1976). Elution was performed in parallel with glycine-NaOH buffer pH 11.5 and 3% beef extract pH 9.0. Reconcentration of the glycine eluates by



membrane adsorption and of beef extract eluates by flocculation was compared. Five hundred liters of tap water were tested with a mean recovery from the glycine buffer of 35% and with a mean recovery from the organic flocculation of 74.4%.

Glycine eluates have been reconcentrated by adsorption to a floc formed at pH 7.0 using  $\text{AlCl}_3$  or pH 3.5 using  $\text{FeCl}_3$  (Payment et al., 1976). Following separation of the floc, virus was recovered by elution with a small volume of glycine pH 11.5 (for  $\text{AlCl}_3$ ) or fetal calf serum (FCS) (for  $\text{FeCl}_3$ ). The formation of a second floc followed by elution of virus was necessary for the  $\text{AlCl}_3$  procedure. Recoveries of virus using either procedure averaged 54%.

The process of hydroextraction exposes a sample to a macromolecular hydroscopic material across a semipermeable membrane. Water, but not virus, passes through the membrane, resulting in a reduction of sample volume. Cliver (1967) used hydroextraction to concentrate 100 ml virus samples to 2 ml in 2 to 3 h using PEG 20,000. Virus recoveries varied from 10 to 30%. Shuval et al. (1967) effected a 100 fold concentration of 1 l virus suspensions within 18 h. Virus recoveries ranged from 20 to 50%. The major loss of virus was attributed to its adsorption to membrane surfaces.

The first fish virus to be isolated was the causal agent of IPN, a disease of salmonids (Wolf et al., 1960). Infectious pancreatic necrosis is acute, highly contagious

and can result in high mortality of young hatchery fish in the fry and fingerling stages. Survivors of an infection remain infected and become adult carriers. The disease initially was believed to be restricted to North America. It spread to France in 1965 through a shipment of infected eggs from the United States (Wolf, 1966b). Since that time isolations from Denmark and Sweden (Jorgensen and Bregnballe, 1969), Italy and the United Kingdom (Wolf, 1972), and Japan (Sano, 1971) have been documented.

Infectious pancreatic necrosis virus was classified as a picornavirus based upon its nucleic acid type and size. It was reported to be 18.5 nm in diameter (Cerini and Malsberger, 1965) and to contain RNA (Malsberger and Cerini, 1965). As more information became available this classification appeared incorrect and the virus was reclassified as a reovirus. Recently, it has been suggested that the IPN virus may belong to a totally new group of viruses (Kelly and Loh, 1972; Dobos, 1976). The controversy arose over conflicting reports on virion size and nucleic acid strandedness. Electron microscopic examination of negatively stained virus have described virion size as 65 nm (Moss and Gravell, 1969), 74 nm (Kelly and Loh, 1972), and 70 nm (MacDonald and Yamamoto, 1977). Virus containing single stranded RNA has been described based on metabolic inhibition (Malsberger and Cerini, 1965), cytochemical and autoradiographic studies (Nicholson, 1971) and action of pancreatic RNase, base composition and thermal

denaturation (Kelly and Loh, 1972). Virus containing double stranded RNA has been described based upon thermal denaturation (Argot, 1969), pancreatic RNase, density in  $\text{Cs}_2\text{SO}_4$ , thermal denaturation and base composition (Cohen et al., 1973), polyacrylamide gel electrophoresis, Kleinschmidt protein film technique and RNase (MacDonald and Yamamoto, 1977). Conflicting reports on acridine orange staining of infected cells also exist. The absence of pale green inclusion bodies was reported by Nicholson (1971) who used a Rainbow trout gonad cell line (RTG-2) infected with IPN virus. Argot and Malsberger (1972) reported occasional areas of green fluorescence in a blue gill fibroblastic cell line (BF-2) infected with IPN virus. Kelly and Loh (1975) reported the presence of red-orange stained inclusion bodies late in the infectious cycle of RTG-2 and swordtail (SWT) cells infected with IPN virus at multiplicities of  $10 \text{ TCID}_{50}$  per cell.

Infectious pancreatic necrosis virus is extremely cytolytic and easily detected in susceptible cell cultures using established fish cell lines. Isolation from infected fish during an outbreak is not difficult but isolation from a carrier fish may be. Clinical diagnosis is made on the basis of characteristic behavior such as distinctive spiral swimming. Pathological diagnosis is made on the basis of histological findings (Wolf and Quimby, 1967). Positive identification involves the use of cell cultures and serum neutralization tests (Amend and Wedemeyer, 1970) or

immunofluorescent assay (Argot, 1969; Piper et al., 1973; Tu et al., 1974). Infectious pancreatic necrosis virus has been isolated from a variety of fish tissue including kidneys, ovarian fluid, eggs, and peritoneal washings. Complete guidelines for virological examination have been reported by Wolf (1970).

## MATERIALS AND METHODS

### Cell Cultures

A chinook salmon embryonic cell line (CHSE) was obtained from Dr. T. Yamamoto, University of Alberta and an RTG-2 cell line was acquired from the American Type Culture Collection (ATCC). Growth media used was Minimal Essential Medium, Hanks balanced salt solution (MEM-Hanks), supplemented with 10% fetal calf serum (FCS), and containing 100 units potassium penicillin G and 100 ug streptomycin sulfate per ml. The maintenance medium was MEM-Hanks with 2% FCS. The cultures were grown routinely in Blake bottles at 18 C and a versene-trypsin solution was used for cell transfers as described by Wolf and Quimby (1973b).

A Buffalo green monkey kidney cell line (BGM) was acquired from Dr. G. Berg, Environmental Protection Agency. Growth medium was MEM, Earles balanced salt solution (MEM-Earles) supplemented as above. Maintenance medium was Melnicks B. The cultures were grown in roller bottles at 37 C and a 0.1% versene-0.125% trypsin solution was used for cell transfers.

### Stock Virus

Infectious pancreatic necrosis strain VR299, obtained from the ATCC, was used throughout this study.

Cell monolayers approximately 75% confluent were used for preparation of stock virus. When advanced cytopathic effects (CPE) were observed, cellular debris was removed by centrifugation at 650 x g for 10 min; supernatants were collected; and the virus-containing supernatant fluid frozen in 5 ml aliquots at -70 C.

#### Virus Assays

An assay based on CPE was carried out in Micro Test II plastic plates (Falcon Plastics). Each well of the plate was seeded with  $2 \times 10^4$  cells per 0.2 ml growth medium; the plate was sealed with sterile tape and incubated at 18 C until monolayers were 75% confluent. Dilutions of virus were made in maintenance medium, monolayer growth medium was removed and 0.2 ml virus dilution inocula introduced. Ten wells per dilution were used. Cytopathic effects were recorded daily for 8 days and the 50% tissue culture infective dose (TCID<sub>50</sub>) calculated according to Reed and Muench (1938).

Isolation of natural virus was attempted using CPE. Five-tenths milliliter of each sample to be tested was added to a monolayer of CHSE or RTG-2 cells in a plastic flask (25 cm<sup>2</sup>, Falcon Plastics) and adsorbed for 30 min at 18 C. The monolayer was overlaid with maintenance medium and development of CPE was followed for 21 days.

The plaque assay was a modification of the method

described by Wolf and Quimby (1973a). A 3 day incubation period at 18 C was used and plaques were visualized with a light source to avoid staining with crystal violet.

Isolation of natural virus was also attempted using this plaque assay by adding 0.5 ml of sample per petri dish. Plaque development was followed until disintegration of the monolayer at approximately 10 days.

A plaque assay for poliovirus type 2 in BGM monolayers in 1 oz prescription bottles (Brockway Glass) was used. Serial tenfold dilutions were made in phosphate buffered saline (PBS) and 0.2 ml inoculated onto the monolayers. After a 1 h adsorption period at room temperature monolayers were overlaid with 5 ml of agar overlay. Plaques were counted daily for 5 days.

#### Cell-Associated Virus

The possibility of cell-associated virus was evaluated. A 5 ml sample of virus plus monolayer cellular debris was frozen and thawed three times. Five ml samples also were sonicated using a MSE 100 watt ultrasonic disintegrator for 2, 3, 4 or 5 min. All virus samples, including a 5 ml control sample of virus plus cellular debris, were harvested as described above and assayed using the TCID<sub>50</sub> system.

### Sample Sterilization

Prior to inoculation of cell cultures, samples to be tested were sterilized by ethyl ether treatment. One ml of anesthetic grade ether per 4 ml of sample was added, mixed thoroughly and held at 4 C for 12-18 h. The sample was then transferred to a sterile petri dish. Ether was removed during a 2 h evaporation period on ice within a Baker Sterigard cabinet, with the petr dish cover slightly ajar.

### Stability of IPN Virus to Change in pH

Infectious pancreatic necrosis virus added to 15 ml volumes of 0.05 M glycine adjusted to pH 4.0, 5.0, 6.0, 8.0, 9.0, 10.0 and 11.0 was assayed using TCID<sub>50</sub> assay after 0, 0.5, 1.0, 2.0, 4.0 and 24.0 h contact times. Test suspensions were mixed, 2 ml withdrawn and the pH neutralized by the addition of 4 ml of maintenance medium with Hepes buffer, final concentration 25 mM.

The effect of acid adjustment to pH 6.0 was examined. Infectious pancreatic necrosis virus was added to 4 l aliquots of 0.01 M glycine, pH 7.0. After adjustment to pH 6.0 using 0.5 N HCl, 0.1 N HCl or 0.001 N HCl, samples were taken. The samples were sterilized by ether treatment and assayed using TCID<sub>50</sub> endpoints.



### Virus Concentration

Passage of IPN virus through 10 inch Fulflo filters (Carborundum Co., Table 1) was tested at pH 7.0. Each filter was rinsed 3 times with 19 l of distilled water to remove residual detergent and blown dry with N<sub>2</sub>. Infectious pancreatic necrosis virus (approximately  $1 \times 10^3$  TCID<sub>50</sub>/ml) was added to 4 l of 0.01 M glycine and allowed to mix for 5 min. After removal of a sample for input virus measurement, test suspensions were passed through filters at flow rates of 380 ml/min. A sample of final filtrate was taken and assayed.

The effect of clay and salmonid feces on the nylon (N39) filter chosen for clarification of water samples was evaluated. Infectious pancreatic necrosis virus was added to 4 l of distilled water, and clay or feces at 0.2 gm/l added. The suspension was passed through the filter at flow rates of 380 ml/min. Samples before and after clay addition as well as filtration were used to determine virus response to clay and filtration treatments.

### Virus Adsorption to Filters

Retention of virus by filters was studied using 10 inch Fulflo filters (Table 1). Test virus was added to 4 l of 0.01 M glycine and the pH adjusted to 3.5, 4.5 or 6.0 with 0.5 N HCl. Tests were carried out at pH 3.5 with final

Table 1. Carborundum Filter Designations

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<u>Code</u>	<u>Type of Material</u>
019	Orlon
039	Orlon
W10A	Cellulose acetate
K27	Fiberglass
S39	Polyester
39	Cotton
D39	Dynel
E39	Viscose
N39	Nylon
M39	Polypropylene

---

Filters manufactured by Commercial Filters Division,  
Lebanon, Indiana.

concentrations of 0.0005 M  $\text{AlCl}_3$ , or with 0.05 M  $\text{MgCl}_2$  at pH 4.5 and 6.0. Suspensions were mixed for 3 min and input virus samples taken. Flow rates of 380 ml/min were maintained and samples of final filtrates taken for virus tests.

#### Virus Elution from Filters

Noncyclic elution of virus from filters was carried out using 3% beef extract, pH 9.0. Approximately 1 l of beef extract per filter was allowed to remain in contact with the filter for 5 min and then removed by positive pressure. The eluate was neutralized to pH 7.2 with 0.5 N HCl and assayed for virus.

Cyclic elution was performed using a peristaltic pump (Randolph or Cole Palmer). The beef extract was pumped in one direction through filters for 4 min, the direction reversed for an additional 4 min and the eluate removed by positive pressure. Eluates were neutralized to pH 7.2 with 0.5 N HCl and assayed for virus.

#### Reconcentration of Virus Eluates

Concentration of eluates was necessary to further reduce the volume prior to inoculation of cell cultures. Concentration of eluates by ultrafiltration was examined first. Test virus was added to 100 ml of water or beef

extract. The samples were placed in an Amicon ultrafilter unit with a PM30 membrane filter. After reduction of volume to 15 ml the suspensions were assayed for virus.

Beef extract flocculation as described by Katzenelson et al. (1976) was examined using 450 ml of 3% beef extract with test virus added. The sample was divided into 3 equal parts and the pH adjusted to 3.5, 4.0 and 4.5 respectively. The suspensions were mixed for 30 min, followed by centrifugation at 3000 x g for 10 min. The supernatant fluid was discarded and the precipitate redissolved in 7.5 ml of  $\text{Na}_2\text{HPO}_4$  (0.15 M). The final pH was 7.2 and the samples were assayed for virus.

Two aqueous polymer two-phase systems (Fields, 1974) were evaluated, each consisting of two parts. In system A, all of the following reagents were added dry, one at a time while slowly mixing: NaCl to 0.05 M, sodium dextran sulfate 2000 to 0.2% and polyethylene glycol 4000 to 6.43%. The solution was allowed to mix for 1 h at 4 C and placed in a separatory funnel for 18 h at 4 C. After development of the two phases the bottom phase and interphase were collected and the top phase discarded. The procedure was continued by slowly adding dry NaCl to a final concentration of 1 M and vortexed thoroughly. After 18 h at 4 C and development of two phases, followed by centrifugation at 120 x g for 10 min, the top and interphase portions were removed. Samples were etherized and assayed for virus.

In system B, to 1 l of eluate, pH 7.2, the following

reagents were added, one at a time, while slowly mixing; dry NaCl to 0.15 M, 26.2 g of 20% (w/w) dextran 2000, 4.8 g methyl cellulose dissolved in 40 g of hot 5 M NaCl (20 g distilled water to rinse out the slurry). The solution was allowed to mix for 1 h at 4 C and placed in a separatory funnel for 48 h at 4 C. After development of the two phases, the bottom and interphase portions were collected and an equal volume of 10% (w/w) polyethylene glycol 6000 was added. After 1 h mixing at 4 C, the solution was placed in a separatory funnel and the two phases allowed to develop for 24 h at 4 C. Bottom and interphase portions were withdrawn and, after etherization, assayed using the TCID<sub>50</sub> system.

#### Model System

A model system was established based on the results of the methods described. Samples consisting of 190 l of nonchlorinated tap water containing test virus were passed through a nylon (N39) filter. Filtrates were adjusted to pH 6, and passed through two viscose (E39) filters and a 293 mm Cox membrane filter assembly composed of 2.0 and 0.45 um filters. Flow rates were 380 ml/min. A 3% beef extract, pH 9, eluent was used for recovery of virus. Eluates were reconcentrated by aqueous polymer two-phase system A.

### Field Studies

Field trials were carried out at the Berlin National Fish Hatchery, Berlin, New Hampshire. Samples consisting of 380 l were collected at two sites supplied from independent water sources. One site, designated A, was sampled at the end of 20 raceways while the second site, designated B, was sampled at the end of 18 raceways. There were six field trials: October 1975, and May, June, July, August, and September, 1976.

### Serum Neutralization Tests

Natural virus isolates were identified by micro serum neutralization tests using a seven strain IPN polyvalent antiserum obtained from Dr. Ken Wolf of the Eastern Fish Disease Laboratory, Fish and Wildlife Service, U.S. Department of the Interior, Kearneysville, West Virginia. The antiserum was titered using stock IPN virus (VR299) diluted in PBS to give a final concentration of 100 TCID per 0.025 ml. Infectious pancreatic necrosis antiserum was added in undilute, 1:5, 1:25, 1:125, 1:625 dilutions. Controls with normal rabbit serum in place of IPN antiserum were used in parallel tests. Virus controls of 0.025 ml PBS in place of antiserum were also included. The mixtures were allowed to react for 30 min at 18 C and then transferred to MicroTest II plates containing CHSE

monolayers with growth media removed. The mixtures were then adsorbed for an additional 30 min at 18 C and the monolayers were overlaid with 0.2 ml maintenance media and observed for development of CPE for 7 days. All tests were made in triplicate. Titrations of the virus dilution used were made to determine the exact amount of virus added.

Isolates chosen for serum neutralization tests were re-passaged to increase virus titers. Three samples were assayed using the standard TCID<sub>50</sub> system and all dilutions of virus were based on the titers obtained. The actual serum neutralization test consisted of 100 TCID<sub>50</sub> per 0.025 ml plus 0.025 ml of 20 antibody units of the polyvalent antiserum. Controls included 100 TCID<sub>50</sub> units per 0.025 ml plus 0.025 ml of normal rabbit serum (dilution equal to IPN virus antiserum) and 100 TCID<sub>50</sub> units per 0.025 ml plus 0.025 ml PBS. All test were performed in duplicate. The virus-antiserum was allowed to mix for 30 min at 18 C and then transferred to MicroTest II plates containing CHSE monolayers with growth media removed. After adsorption to monolayers for 30 min at 18 C, each well was overlaid with 0.2 ml of maintenance media and the assay read at 3 days.

#### Electron Microscopy

Viral suspensions clarified only by centrifugation and containing approximately  $1 \times 10^6$  TCID<sub>50</sub>/ml were adsorbed for 3 min to 400 mesh formvar coated copper grids. The

suspension was removed by capillary action, the grid rinsed for 5 sec in PBS and 4% phosphotungstic acid pH 7.0 was added for 2 min and removed. The samples were examined using a Phillips Electron Microscope Model 200.



## RESULTS

Assay results obtained from liquid overlays of test monolayers (TCID<sub>50</sub> titer) and agar overlays (PFU) were compared for their sensitivity as a prelude to virus quantitation measurements. The results of four trials are shown in Table 2. The TCID<sub>50</sub> assays were more sensitive than plaquing procedures in all trials. Approximately three times as much virus was measured by TCID<sub>50</sub> assays regardless of the number of infectious units used. The results of the comparative tests suggested a TCID<sub>50</sub> assay would be the procedure of choice whether large or small numbers of viruses were involved.

The time needed for completion of TCID<sub>50</sub> assay results was determined in a series of trials in which titers were calculated over a period of 8 successive days. The results of three representative trials are shown in Table 3. Titrations were complete at 4 days in trial 3, at 5 days in trial 1 and at 7 days in trial 2. Attainment of completed titrations by day 5 in two of the trials, and 92 percent completion in the third suggested that a 5 to 7 day period would prove adequate for completion of most TCID<sub>50</sub> assays.

Assay accuracy was examined in a series of trials in which the degree of variability of assays performed in four replicates was determined. The results of four representative trials are given in Table 4. Assay reproducibility was judged to be good with variation

Table 2. Quantitative Measurements of Infectious Pancreatic Necrosis Virus Numbers by Plaque and TCID<sub>50</sub> Assay

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<u>Trial</u>	<u>PFU/ml</u>	<u>Virus Assay</u> <sup>a</sup>	<u>TCID<sub>50</sub>/ml</u>
1	2.0x10 <sup>6</sup>		7.3x10 <sup>6</sup>
2	3.8x10 <sup>5</sup>		1.4x10 <sup>6</sup>
3	1.6x10 <sup>4</sup>		5.0x10 <sup>4</sup>
4	7.2x10 <sup>2</sup>		2.6x10 <sup>3</sup>

---

<sup>a</sup> Assays were made with CHSE cell culture monolayers.

Table 3. TCID<sub>50</sub> Titers Determined as a Function of Time

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<u>Day</u>	<u>Trial</u> <sup>a</sup>		
	<u>1</u>	<u>2</u>	<u>3</u>
1	1.6x10 <sup>2</sup>	ND	ND
2	1.2x10 <sup>2</sup>	1.4x10 <sup>4</sup>	ND
3	1.6x10 <sup>5</sup>	1.8x10 <sup>4</sup>	5.0x10 <sup>4</sup>
4	2.6x10 <sup>5</sup>	2.1x10 <sup>4</sup>	7.3x10 <sup>4</sup>
5	5.0x10 <sup>5</sup>	2.4x10 <sup>5</sup>	7.3x10 <sup>4</sup>
6	5.0x10 <sup>5</sup>	2.4x10 <sup>5</sup>	7.3x10 <sup>4</sup>
7	5.0x10 <sup>5</sup>	3.1x10 <sup>5</sup>	7.3x10 <sup>4</sup>
8	5.0x10 <sup>5</sup>	3.1x10 <sup>5</sup>	7.3x10 <sup>4</sup>

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ND<sub>a</sub> - Not done  
 Different stock viral suspensions were used for each  
 trial.

Table 4. Variability of Infectious Pancreatic Necrosis  
Virus Assays Determined by TCID<sub>50</sub> Titrations

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<u>Trial</u>	<u>Virus Assay (TCID<sub>50</sub>/ml)</u>			
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
1	2.5x10 <sup>2</sup>	2.1x10 <sup>2</sup>	1.9x10 <sup>2</sup>	2.4x10 <sup>2</sup>
2	1.4x10 <sup>3</sup>	1.5x10 <sup>3</sup>	1.8x10 <sup>3</sup>	1.9x10 <sup>3</sup>
3	5.9x10 <sup>4</sup>	6.0x10 <sup>4</sup>	6.5x10 <sup>4</sup>	6.7x10 <sup>3</sup>
4	1.4x10 <sup>5</sup>	1.4x10 <sup>5</sup>	1.3x10 <sup>5</sup>	9.6x10 <sup>4</sup>

generally less than 15 percent. Assay reproducibility was greatest when large numbers were involved. It was important to know that assay accuracy fell within acceptable limits when low numbers were encountered. The accuracy was acceptable over a TCID<sub>50</sub> range from 250 to 100,000.

Infectious pancreatic necrosis virus has been considered to be cell-associated by many investigators who found it necessary to treat infected cell cultures by procedures causing release of progeny virus from cellular debris. In order to determine a method yielding the largest amount of virus from infected cell cultures, different treatments for release of progeny virus were compared with nontreated viral suspensions. The results are given in Table 5. The treatment by freezing and thawing did not result in a greater release of progeny virus than was found for non-treated control suspensions. Judging from the decreased titers, the freeze-thaw treatment was damaging to virus and resulted in the loss of virus activity. There was little difference between untreated and two and three minute sonication results. A significant loss in titer was found after five minute sonication treatment. This loss probably was caused by damage to virion structures. All viral suspensions that were sonicated were maintained at 4 C. Since sonication treatment did not result in greater release of progeny virus than obtained with untreated suspensions, further use was not considered.

Prior to inoculation onto cell culture monolayers,

Table 5. The Use of Sonication and Freeze-Thaw Treatments for Release of Progeny Virus from Host Cells

<u>Trial</u>	<u>Virus Assay (TCID<sub>50</sub>/ml)</u>						
	<u>Nontreated</u> <sup>a</sup>		<u>Sonication (min)</u> <sup>c</sup>				
	<u>Viral Suspension</u>	<u>Freeze-thaw</u> <sup>b</sup>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>
1	8.7x10 <sup>6</sup>	1.6x10 <sup>6</sup>	5.0x10 <sup>6</sup>	6.4x10 <sup>6</sup>	ND	ND	ND
2	2.1x10 <sup>6</sup>	1.1x10 <sup>6</sup>	1.6x10 <sup>6</sup>	1.8x10 <sup>6</sup>	2.0x10 <sup>6</sup>	1.6x10 <sup>6</sup>	9.6x10 <sup>5</sup>

ND - Not done

<sup>a</sup>Supernatant fluid clarified by centrifugation of cell cultures showing 3 to 4+ CPE.

<sup>b</sup>Cells frozen and thawed three times.

<sup>c</sup>Cells sonicated in MSE ultrasonic disintegrator at 100 watts at 10 C.

samples were freed from bacterial and fungal contaminants. The successful use of ether treatment to remove such contaminants from water samples led to trials to determine the effect of preinoculation ether treatment of IPN virus samples. The results after 18 h of ether treatment are given in Table 6. There was no significant loss of viral infectivity, indicating that ether could be used as a preinoculation "sterilization" treatment for samples containing IPN virus. This confirms the work of Malsberger and Cerini (1963) who found that IPN virus was not significantly affected by ether treatment.

#### Stability of IPN Virus to Changes in pH

Virus adsorption phenomena are pH dependent. Adsorption is favored at lower pH values (3.5 to 6.0), with elution at higher pH values (9.0 to 11.5). Virus stability to changes in pH determine the extent to which suspensions can be manipulated safely over a range from 3.5 to 11.5. It was necessary to determine IPN virus stability to pH change before trials involving the adsorption and elution phenomena could be considered. Samples of test virus suspension were exposed for periods of time from one-half to four hours at pH values from 3 to 11. Virus survival at each trial pH as a function of time was determined by assay. The results of these assays are given in Table 7.

Virus inactivation occurred rapidly at pH 3.0 and

Table 6. Infectious Pancreatic Necrosis Virus Stability to Ether<sup>a</sup>

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<u>Trial</u>	<u>Virus Assay (TCID<sub>50</sub>/ml)</u>	
	<u>Before Ether</u>	<u>After Ether</u>
1	5.5x10 <sup>5</sup>	7.2x10 <sup>5</sup>
2	1.1x10 <sup>4</sup>	1.2x10 <sup>4</sup>
3	2.6x10 <sup>3</sup>	2.4x10 <sup>3</sup>
4	6.2x10 <sup>2</sup>	6.1x10 <sup>2</sup>

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<sup>a</sup>Diethyl ether, anesthetic grade



Table 7. Inactivation of Infectious Pancreatic Necrosis Virus at Acid and Alkaline pH over a Four Hour Interval

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pH	Virus Assay (TCID <sub>50</sub> /ml)				
	Time (h)				
	<u>0</u>	<u>0.5</u>	<u>1.0</u>	<u>2.0</u>	<u>4.0</u>
3	6.0x10 <sup>3</sup>	0	0	0	0
4	6.0x10 <sup>3</sup>	4.7x10 <sup>3</sup>	4.0x10 <sup>3</sup>	1.6x10 <sup>3</sup>	1.6x10 <sup>3</sup>
5	6.0x10 <sup>3</sup>	5.0x10 <sup>3</sup>	4.7x10 <sup>3</sup>	4.7x10 <sup>3</sup>	3.2x10 <sup>3</sup>
6	6.0x10 <sup>3</sup>	6.0x10 <sup>3</sup>	5.8x10 <sup>3</sup>	6.0x10 <sup>3</sup>	6.0x10 <sup>3</sup>
7	6.0x10 <sup>3</sup>	5.8x10 <sup>3</sup>	6.0x10 <sup>3</sup>	6.0x10 <sup>3</sup>	5.8x10 <sup>3</sup>
8	6.0x10 <sup>3</sup>	6.0x10 <sup>3</sup>	6.0x10 <sup>3</sup>	4.0x10 <sup>3</sup>	4.0x10 <sup>3</sup>
9	6.0x10 <sup>3</sup>	6.0x10 <sup>3</sup>	5.0x10 <sup>3</sup>	4.0x10 <sup>3</sup>	4.0x10 <sup>3</sup>
10	6.0x10 <sup>3</sup>	2.1x10 <sup>3</sup>	0	0	0
11	6.0x10 <sup>3</sup>	0	0	0	0

10.0. At pH values within these limits, inactivation was found to be a function of the degree of acidity or basicity plus time of exposure. No significant loss of virus was found at pH 6.0 or 7.0, but the degree of loss above or below this range increased with time. It was judged practical to evaluate adsorptive phenomena at pH 3.5, 4.5 and 6.0 and to carry out elutions at alkaline pH up to 9.0 based upon 1/2 hour stabilities.

### Virus Concentration

#### Clarification

Sampling of large volumes of natural waters often is complicated by the presence of suspended solids which clog filters, reduce flow rates and make further sampling virtually impossible. The use of clarifying filters for removal of suspended solids was considered, recognizing that solids-adsorbed virus would be lost if the clarifying filters were not treated for recovery of virus. These filters were evaluated for their ability to allow passage of freely suspended IPN virus at pH 7.0. The results are given in Table 8. Several filters such as the nylon (N39), cellulose acetate (W10A) and dynel (D39) varieties allowed the greatest proportion of test virus to pass. Of these the nylon (N39) filter was chosen for clarification use because it consistently allowed passage of 100% of IPN virus.

Table 8. Passage of Infectious Pancreatic Necrosis Virus Through Filters at pH 7.0

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<u>Filter</u>	<u>Composition</u>	<u>Virus in Filtrate (percent)</u>
019	Orlon	ND
039	Orlon	85
W10A	Cellulose acetate	98
K27	Fiberglass	ND
S39	Polyester	84
39	Cotton	48
D39	Dynel	90
E39	Viscose	0
N39	Nylon	100
M39	Polypropylene	33

---

ND - Not done

The nylon (N39) filter was next evaluated for its ability to permit passage of IPN virus in water containing a clay or finely subdivided fecal material. The results are given in Table 9. Most virus passed through the nylon (N39) filters in the presence of either kaolinite or fish feces. Most of the solids-adsorbed virus was recovered on elution of the filters. Total recoveries of virtually all of the test IPN virus suggested clays or fish fecal material suspended in water being sampled would not interfere with virus recovery efficiency when nylon (N39) clarifying filters were used, provided elution of these filters was carried out.

#### Adsorption

Enterovirus is efficiently adsorbed to filters at pH 3.5 in the presence of 0.0005 M  $AlCl_3$  or at pH 4.5 in the presence of 0.05 M  $MgCl_2$ . Optimal conditions for adenovirus adsorption occur at pH 6.0 in the presence of 0.05 M  $MgCl_2$ . Using samples of 4 l of distilled water with added virus, examination of the effect of pH and/or added salt on the retention of IPN virus by different filters was made. The results of several trials at excess hydrogen ion concentrations are given in Table 10. Filters that retained all or most of the test virus were candidates for use as adsorbing filters. Conversely filters retaining little or no virus were potential candidates for use as clarifying

Table 9. Infectious Pancreatic Necrosis Virus Recovery from Nylon Filters in the Presence of Interfering Suspended Solids

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<u>Suspended Solid</u>	<u>Virus Recovery (percent)</u>		
	<u>Filtrate</u> <sup>a</sup>	<u>Filter</u> <sup>b</sup>	<u>Total</u>
Kaolinite	81	18	99
Salmonid feces	88	10	98

---

<sup>a</sup> Virus passing through filter and appearing in filtrate.

<sup>b</sup> Virus recovered from filter by elution.

Table 10. Adsorption of Infectious Pancreatic Necrosis Virus to Test Filters

Sample <u>Treatment</u>	Virus Retention by Test Filters <u>(percent Adsorption)</u>									
	<u>019</u>	<u>039</u>	<u>W10A</u>	<u>K27</u>	<u>S39</u>	<u>39</u>	<u>D39</u>	<u>E39</u>	<u>N39</u>	<u>M39</u>
pH 3.5	0	13	97	97	ND	ND	ND	95	79	55
pH 3.5 plus AlCl <sub>3</sub>	ND	71	100	100	0	100	100	66	0	0
pH 4.5	0	44	86	0	100	29	33	79	75	95
pH 4.5 plus MgCl <sub>2</sub>	93	100	0	100	41	100	35	100	100	100
pH 6.0	13	10	9	76	15	0	ND	100	0	0
pH 6.0 plus MgCl <sub>2</sub>	34	65	97	94	100	93	0	90	0	37

ND - Not done

filters. Both retention and nonretention of virus by test filters was influenced by pH and enhancing salt. Several test result patterns were found. Filters adsorbing virus at acid pH, with enhancement of adsorption in the presence of  $Al^{+++}$  or  $Mg^{++}$  ions were regarded as filters to be used for adsorption of virus. Cellulose acetate (W10A), fiberglass (K27) and cotton (39) filters were examples identifiable as adsorbent filters. Failure to obtain enhancement of adsorption in the presence of  $Al^{+++}$  or  $Mg^{++}$  ions represented a pattern identifiable with a clarifying filter. Nylon (N39) and orlon (O19) filters were examples of this pattern of test results. Still another pattern was found with viscose (E39) filters. Based on virus adsorption at acid pH, with or without enhancement in the presence of salt, these filters could be considered adsorptive in type. Dynel (D39) and polypropylene (M39) filters could not clearly be identifiable as suitable for either adsorption or clarification functions.

Selection of adsorbing filters for recovery of IPN virus from aqueous suspensions was influenced by viral pH stability. As shown previously IPN virus stability at acid pH less than 6.0 declined significantly with time. The report that some natural isolates of IPN virus are not stable at a pH varying widely from neutrality (Wolf, 1972) led to the designation of pH 6.0 as optimal for adsorption. This represented the basis on which a filter would be regarded as an adsorbent filter. The data in Table 10

indicated cellulose acetate (W10A), fiberglass (K27), polyester (S39), cotton (39) or viscose (E39) filters could be used for adsorption of virus; viscose (E39) filters were chosen.

Virus inactivation during acid pH adjustments was tested using hydrochloric acid solutions with normalities of 0.5, 0.1 and 0.001. Aqueous virus suspensions were adjusted to pH 6.0 using the three acid normalities. Virus inactivation ranged from 17% with 0.5 N HCl, to 6% with 0.1 N HCl, to none with 0.001 N HCl. It was necessary to reconcile the need for dilute acid with practical considerations involving volume of reagent to be added and time required for pH adjustment in the model studies. Solutions of 0.5 N acid were used with recognition of virus inactivation rates. In an effort to eliminate this loss field studies in which pH adjustments were necessary, were carried out with 0.001 N acid injected by means of the Johansen reciprocating pump.

#### Elution

Virus adsorbed to filter surfaces was recovered by elution of the filter with a suitable eluent. Eluent choice was restricted by stability of IPN virus to pH changes. Instability of these viruses to a pH more alkaline than 9.0 eliminated any possibility of selecting the 0.05 M glycine, pH 11.5 eluent used successfully for elution of



enteroviruses from collecting filters. Attention was directed to procedures using proteinaceous solutions adjusted to a pH not greater than 9.0. Three percent beef extract solution at pH 9.0 seemed to be an effective eluent, but the manner in which the elution procedure was carried out was found to be crucial to elution effectiveness. Circulation of eluent through a filter by means of a peristaltic type pump was shown to be more effective than simple contact of eluent with filter surfaces. A comparison of the effectiveness of cyclic versus non-cyclic elution procedures is given in Table 11. The superiority of the cyclic elution procedure was shown in each of the three trials. Averages of 69 versus 22% respectively for cyclic and non-cyclic elutions emphasized the greater effectiveness of the cyclic elution method for recovery of virus.

#### Reconcentration

The need to use liter volumes per filter for recovery of virus produced eluate volumes too great to test directly for virus in cell cultures. It was necessary to reduce or reconcentrate eluates to smaller volumes, all of which could be tested for virus. Reconcentration methods evaluated for their virus recovery effectiveness included ultrafiltration, beef extract flocculation and two aqueous polymer two-phase systems. The results of studies of the effectiveness of reconcentration methods for recovery of

Table 11. A Comparison of the Effectiveness of Cyclic and Non-cyclic Procedures for Recovery of Infectious Pancreatic Necrosis Virus from Viscose Filters

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<u>Trial</u>	Virus Recovery (percent)	
	<u>Cyclic Elution</u>	<u>Non-cyclic Elution</u>
1	53	25
2	74	15
3	80	26
Average	69	22

---

Eluent - 3% beef extract, pH 9.0

test virus from 1 l volumes of 3% beef extract are shown in Table 12.

The most effective reconcentration method was the aqueous polymer two-phase system A. Its effectiveness was based on both the volume reduction and virus recovery obtained. Beef extract flocculation at pH 3.5 was effective for recovery of virus and although only a 20x volume reduction was obtained in the study, greater volume reductions were possible using this method. The greatest draw-back to this method involved exposure of virus to a pH of 3.5 for periods of an hour. This pH was very close to limits of pH stability of the stock virus and probably beyond the pH stability range of most natural IPN virus strains. Ultrafiltration and aqueous polymer two-phase system B methods were ineffective for recovery of virus. Ultrafiltration in addition was incapable of an effective reduction of test sample volume.

#### Model System

A model system was developed based on the series of studies on equipment and methods necessary for the effective recovery of test IPN virus from water. Tests of the effectiveness of this system were based on trials in which IPN virus was added exogenously to 190 l samples of non-chlorinated tapwater. The 190 l samples were passed through a nylon (N39) clarifying filter without prior

Table 12. The Effectiveness of Reconcentration Methods for Recovery of Test Virus from 3 percent Beef Extract Solutions

---

<u>Method</u>	<u>Volume Reduction</u>	<u>Virus Recovery (percent)</u>
Ultrafiltration	10X	21.9
Beef Extract Flocculation pH 4.5	20X	35.7
pH 4.0	20X	67.9
pH 3.5	20X	96.4
Aqueous Polymer 2-Phase System A	200X	100.0
Aqueous Polymer 2-Phase System B	200X	66.0

adjustment of pH. Clarified samples were adjusted to pH 6.0; salt was or was not added to a final concentration of 0.5 M  $MgCl_2$ . Samples were then passed through two viscose (E39) filters and a 293 mm Cox membrane filter at flow rates of 380 to 760 ml/min. A 3% beef extract solution, pH 9.0, was used to recover virus. Eluates were reconcentrated by aqueous polymer two-phase system A. The first trial involved no added salt and used noncyclic elution. The total recovery was 2% with a virus breakthrough of 22%. Seventy-six percent of the original added virus remained unaccounted for suggesting that removal of virus from the adsorbing filters was not satisfactory.

The second trial used a peristaltic pump for cyclic elution. Total recovery increased to 26% but an increase in breakthrough to 35% was also found. Cyclic elution increased the recovery of added virus with only 39% unaccounted for.

The third trial was in the presence of 0.05 M  $MgCl_2$  and used cyclic elution. Total recovery was 24%. A virus breakthrough of 30% occurred. These results suggested there was no significant difference between pH 6.0 and pH 6.0 in the presence of  $Mg^{++}$  in water containing no suspended material.

The chance of viruses other than IPN interfering with recovery of IPN virus was investigated. Poliovirus type 2 and IPN virus were added simultaneously to 190 l of nonchlorinated tapwater and the model system used to

determine rates of recovery of the two viruses. Poliovirus assays were made on BGM monolayers and IPN virus assays on CHSE monolayers. Prior tests had shown neither virus was able to replicate on monolayers specific for the other virus. Poliovirus did not react favorably in this virus concentration system as most of the virus passed through, or if adsorbed, was not eluted from the filters. Less than 1% of the IPN virus adsorbed to the nylon (N39) clarifying filter, but of that 83% was recovered. The two viscose (E39) adsorbing filters retained 66% of the test virus and of that 79% was recovered on elution. The total of input IPN virus recovered from both filters equalled 52%. The presence of another virus such as poliovirus type 2 appeared to have little effect on the recovery of IPN virus.

#### Isolation of Natural Virus

The first field study in October 1975 consisted of two 380 l trials, one at site A and one at site B. The virus concentration system of filters tested at each site consisted of a nylon (N39) filter to clarify the water, two viscose (E39) filters and a 293 mm Cox (epoxy-fiberglass) filter assembly (2.0 mu and 0.45 mu) to adsorb virus. The pH of the water was 6.2; no pH adjustment was made and no salt was added. Cyclic elution of clarifying and adsorbent filters was carried out with 3% beef extract, pH 9.0 as the eluent. For these and all other field studies, the filters

were eluted separately, samples were taken from the eluates and aqueous polymer two-phase system A was used to further concentrate the eluate. All samples were either sterilized and tested for the presence of natural IPN virus using CHSE and RTG-2 cell monolayers with both the CPE and plaquing methods described. No isolates were obtained from either trial.

The second field study was made in May 1976. Two trials were carried out at site B using the same system of filters as in the first field study. The pH in this and all the remaining studies was adjusted to 6.0 with the Johansen proportioner pump. The filters from the first trial were cyclically eluted with 3% beef extract, pH 9.0. Natural virus isolates were obtained from seven samples. The filters from the second trial were eluted with 5X nutrient broth, pH 9.0. Three natural virus isolates were recovered. Only one trial was made at site A because of poor weather. The Cox filter assembly slowed the flow rate to 380 ml/min after passage of 75 l; because of this its use was discontinued in an effort to maintain a flow rate of 380 to 760 ml/min. Cyclic elution was used with 5X nutrient broth as an eluent. Four natural virus isolations were made.

All samples for the third field trial in June 1976 were made at site B. The filter system used for this and all of the remaining studies consisted of nylon (N39) filter (clarifying), two viscose (E39) filters (adsorbing) and a fiberglass (K27) filter (adsorbing). In the model studies,

there was some breakthrough from the viscose (E39) filters and the fiberglass (K27) was chosen as a back-up adsorbing filter. For the first sample no salt was added and the filters were eluted with 3% beef extract, pH 9.0. Three natural virus isolates were recovered. For the second sample,  $MgCl_2$  was added to a final concentration of 0.05 M and the filters were eluted with 3% beef extract, pH 9.0. Thirty-three natural virus isolates were recovered. The third sample was collected without added salt; the filters were eluted with 5X nutrient broth, pH 9.0. Twenty-seven natural virus isolates were recovered. Magnesium chloride in a final concentration of 0.05 M, was used in the collection of the fourth sample; the filters were eluted with 5X nutrient broth, pH 9.0. Twenty-seven natural virus isolates were recovered.

The fourth field trial was made in July 1976. One sample was collected at site A using the system described with no added salt and cyclic elution with 5X nutrient broth, pH 9.0. Seven natural virus isolates were recovered. Four samples were collected at site B. When no salt was present and the filters were cyclically eluted with 3% beef extract, pH 9.0, 22 isolates of natural virus were recovered. When  $MgCl_2$  in a final concentration of 0.05 M was used and cyclic elution with 3% beef extract, pH 9.0 was carried out, 38 natural virus isolates were recovered. When these two samples were repeated with 5X nutrient broth, pH 9.0 as the eluent, and a final concentration of 0.05 M  $MgCl_2$



present, 34 natural virus isolates were recovered. When no MgCl was added 46 natural virus isolates were recovered.

The field trial in August 1976 was conducted at site B following the same experimental design used in July. With no salt present and the filters cyclically eluted with 3% beef extract, pH 9.0, 6 natural virus isolates were recovered. With a final concentration of 0.05 M MgCl<sub>2</sub> and the same eluent in use, 12 natural virus isolates were recovered. With no salt present and the filters cyclically eluted with 5X nutrient broth, pH 9.0, 13 natural virus isolates were recovered. With a final concentration of 0.05 M MgCl<sub>2</sub> and the same eluent in use, 10 natural virus isolates were recovered.

The sixth and final field trial made in September 1976 followed the same experimental design used in the previous two months trials. Two natural virus isolates were recovered with a final concentration of 0.05 M MgCl<sub>2</sub> and cyclic elution of filters using 3% beef extract, pH 9.0. With salt absent and using the same eluent there were 3 natural virus isolates recovered. With no salt present and the filters cyclically eluted with 5X nutrient broth, pH 9.0, 3 natural virus isolates were recovered. In the presence of salt using the same eluent, one natural virus isolate was recovered.

A total of 11 natural virus isolates were recovered from site A. Due to the small number of samples at site A, comparisons of natural virus isolations at sites A and B

could not be made. Figure 1 illustrates the number of virus isolates per 1500 l of hatchery water at site B for each field sample. A large increase in the number of isolates occurred in June and July. The combined results of all the field trials from site B are presented in Table 13. The total number of isolates recovered, in the presence or absence of  $MgCl_2$ , with the use of nutrient broth or beef extract as eluent, and in the eluate or reconcentrated samples is given. While the nature of these experiments did not allow relevant statistical treatment, some combinations of the treatments used were of interest. Use of  $MgCl_2$  led to more virus isolations in three out of five trials. A clear cut advantage for no  $MgCl_2$  treatment was found in one of five trials. One hundred and eighty-four isolates were recovered with  $MgCl_2$ , 140 without  $MgCl_2$ . This indicated  $MgCl_2$  may have enhanced recovery of natural virus.

Comparison of the two eluents shows 130 isolates were recovered with beef extract and 194 with nutrient broth. This difference was the result primarily of recoveries made in June, when 48 more isolates were associated with the use of nutrient broth. With the exception of this one month, there was no appreciable difference in effectiveness between the two eluents. Comparisons of virus recoveries with and without reconcentration of samples showed more isolations were made with reconcentration in four out of five trials where positive results were obtained. The greater total of

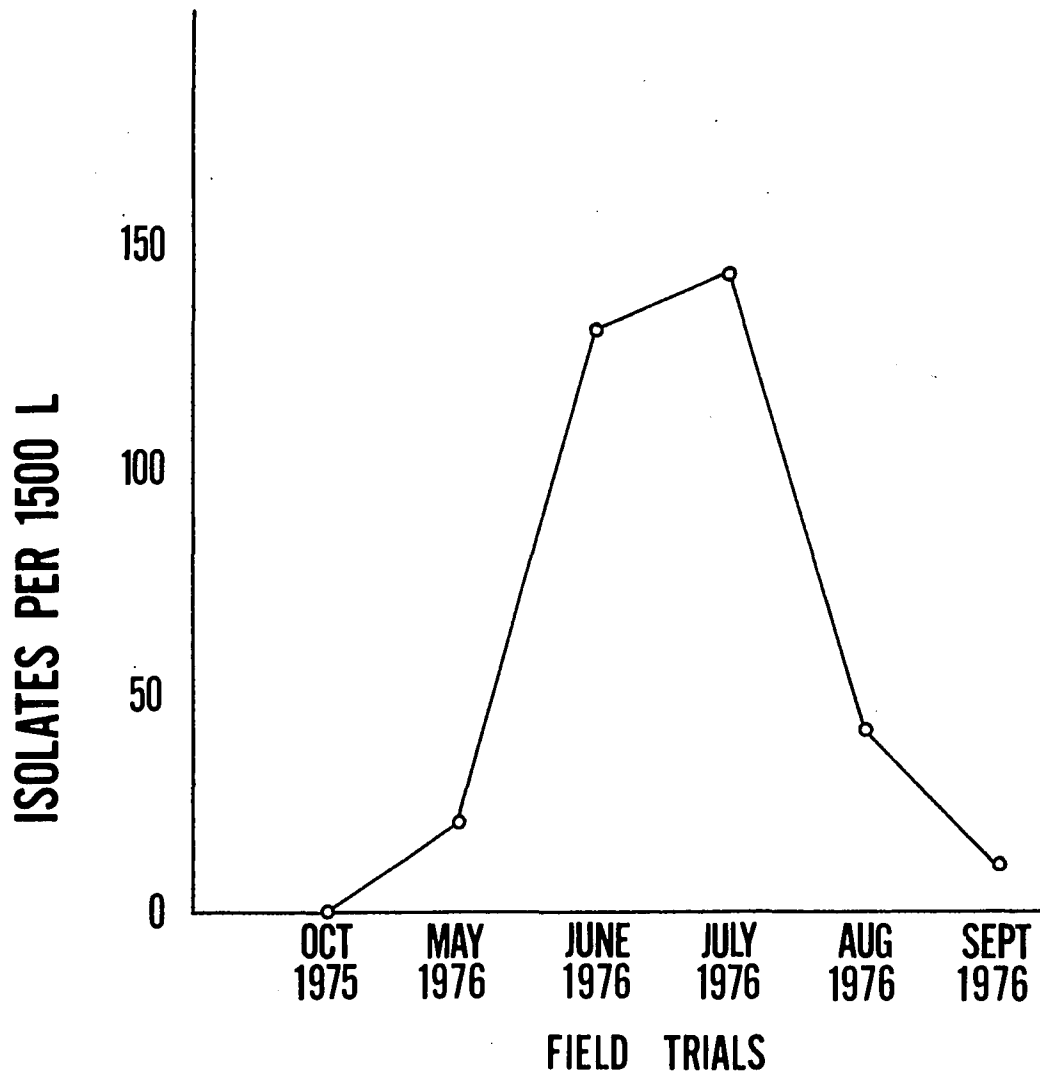


Figure 1. Infectious Pancreatic Necrosis Virus Isolations Made at Site B, National Fish Hatchery, Berlin, New Hampshire, During 1975 and 1976.

Table 13. Natural Virus Isolates Recovered from Site B

<u>Field Trial</u>	<u>Number of Isolates</u>						<u>Total</u>
	<u>With MgCl<sub>2</sub></u>	<u>Without MgCl<sub>2</sub></u>	<u>With Beef Extract</u>	<u>With Nutrient Broth</u>	<u>With 2-Phase Separation</u>	<u>Without 2-Phase Separation</u>	
Oct 1975	ND	0	0	ND	0	0	0
May 1976	0	10	7	3	10	0	10
June 1976	78	50	40	88	39	89	128
July 1976	80	56	60	76	80	56	136
Aug 1976	22	19	18	23	29	12	41
Sept 1976	4	5	5	4	6	3	9
<u>Total</u>	<u>184</u>	<u>140</u>	<u>130</u>	<u>194</u>	<u>164</u>	<u>160</u>	<u>324</u>

isolations made without reconcentration was the result of a single months isolations in which more than twice as many recoveries were made without reconcentration treatment.

The pattern of results obtained suggested the combination of treatments most advantageous for recovery of natural IPN virus from hatchery or other waters should include use of  $MgCl_2$  for enhancement of adsorption, nutrient broth for elution of filters and reconcentration for preparation of final samples.

Recovery of natural virus was favored by cell culture selection and procedures as well as sample collection procedures. Two fish cultures, CHSE and RTG-2, were used in the studies. Both were used with agar (PFU) and liquid (CPE) overlay procedures. Comparisons of the isolations made using the two cell cultures in conjunction with the two overlay procedures are given in Table 14. The pattern of results favored the use of CHSE monolayers with liquid overlay as the combination most advantageous for recovery of natural virus. This was shown by the total of 199 isolates which were recovered with CHSE monolayers during five of the six field trials, compared to 125 with RTG-2 monolayers. Similarly 210 isolates were recovered by means of CPE procedures compared with 114 isolates recovered by the plaquing method.

Virus recoveries from each of the four filter types used in the field trials were compared to determine their relative effectiveness. The results of these studies are

Table 14. Natural Virus Isolates Recovered from Site B  
According to Host Cell and Isolation Procedure

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<u>Field Trial</u>	<u>Host Cell</u>		<u>Isolation Procedure</u>		<u>Total</u>
	<u>CHSE</u>	<u>RTG-2</u>	<u>CPE</u>	<u>PFU</u>	
Oct 1975	0	0	0	0	0
May 1976	8	2	7	3	10
June 1976	101	27	78	50	128
July 1976	61	75	100	36	136
Aug 1976	20	21	20	21	41
Sept 1976	9	0	5	4	9
Total	199	125	210	114	324

shown in Table 15. Three hundred twenty-four isolates were recovered in six field trials. Two hundred thirty-three of the isolates, or 72% of the total, were recovered from one or more of the three adsorbing filters used. Ninety-one, or 28% of the total, were recovered from the nylon (N39) clarifying filters. The 91 isolates recovered from the nylon clarifying filters were considered to represent solids associated adsorbed virus. These isolates were retained by clarifying filters as a result of virus adsorption to suspended solids present in the water sampled and entrapment of these solids on clarifying filter surfaces. The recovery of these viruses from clarifying filters demonstrated the need to elute clarifying as well as adsorbing filters in order to maximize virus recovery potentials.

Virus recoveries from viscose (E39) and fiberglass (K27) adsorbing filters were virtually identical and neither filter could be considered superior to the other. Recovery of virus from fiberglass (K27) filters in trials where these filters were located downstream from viscose (E39) filters was considered evidence of virus breakthrough from the viscose (E39) filters.

The efficacy of the Cox membrane filters for collection of virus could not be determined due to the very few field trials in which they were used. These filters were not used after the May field trials because their tendency to become plugged with finely divided particulate matter prevented sampling of large volumes of water. The

Table 15. Natural Virus Isolates Recovered on Individual Filters at Site B

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<u>Field Trial</u>	<u>Filter</u>				<u>Total</u>
	<u>N39</u>	<u>E39</u>	<u>Cox</u>	<u>K27</u>	
Oct 1975	0	0	0	ND	0
May 1976	1	5	4	ND	10
June 1976	37	40	ND	51	128
July 1976	42	47	ND	47	136
Aug 1976	8	18	ND	15	41
Sept 1976	3	6	ND	0	9
Total	91	116	4	113	324

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ND - Not done



optimal filter system for recovery of natural virus consisted of a nylon (N39) clarifier, followed by viscose (E39) and fiberglass (K27) filters in series.

#### Natural Virus Isolation from Fingerling Trout

Two fingerling trout displaying typical symptoms of IPN were taken from site B at the hatchery in August 1976. They were subjected to two virus isolation procedures modified from Amend and Wedemeyer (1970). The head and tail were removed and each fish was weighed and treated separately. A 1:50 (w/v) dilution with PBS was followed by homogenization using an omnimixer for 60 sec. To one half of each sample 5X volume of 3% beef extract, pH 9.0 was added, allowed to mix for 5 min and centrifuged at 3000 x g for 10 min. The supernatant was neutralized to pH 7.0, ether sterilized and tested for natural virus. Four isolates each were obtained from fingerling 1 and 2 using this procedure. The remaining half of each sample was filtered using a 0.45 um cellulose nitrate Amicon filter, the filtrate sterilized and tested for natural virus. Two isolates were obtained from fingerling 1 and three isolates from fingerling 2. The isolates displayed typical IPN virus destruction in cell cultures and were confirmed as IPN by serum neutralization tests.

### Serum Neutralization Tests

All "isolates" obtained displayed cell destruction typical of IPN virus. In order to establish that these isolates were IPN virus micro serum neutralization tests were performed using a 7-strain polyvalent IPN antiserum (received from Dr. Ken Wolf).

The antiserum was received untitered and it was titered in a neutralization test using stock IPN virus. The results of the antiserum titration are given in Table 16.

One antibody unit for both strains of IPN virus used was defined as 0.025 ml of a 1:125 dilution. The serum dilutions used, as recommended by Dr. Wolf, represented 5-fold increments. Due to the difference between each dilution, the endpoints of the antibody titrations could not be distinguished more precisely than the 5-fold dilution scheme allowed. The actual titration endpoint may have been closer to the 1:625 dilution than the 1:125 dilution. Definition of 1 antibody unit as 0.025 ml of a 1:125 dilution would be conservative by a factor of as much as 3 to 4 fold if this assumption were true. The ten antibody units calculated for use in the serum neutralization tests could actually have varied between the limits of 10 to 40 units.

It was not feasible to test all of the 324 natural virus isolates in serum neutralization tests to determine

Table 16. The Antibody Titer of a Polyvalent Infectious Pancreatic Necrosis Virus Antiserum

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<u>IPN Virus</u>	<u>IPN Antiserum Dilutions</u>	<u>Neutralization of Virus<sup>a</sup></u>	<u>Normal Serum Dilution</u>	<u>Neutralization of Virus<sup>a</sup></u>
VR299	Undilute	+	Undilute	-
	1:5	+	1:5	-
	1:25	+	1:25	-
	1:125	+	1:125	-
	1:625	-	1:625	-
FR21	Undilute	+	Undilute	-
	1:5	+	1:5	-
	1:25	+	1:25	-
	1:125	+	1:125	-
	1:625	-	1:625	-

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<sup>a</sup>+ = neutralization of test virus. - = no neutralization of test virus.

their IPN identity. Isolates to be tested were chosen with the idea of as equitable a distribution as possible among the several variables represented in the field samples. The distribution of the isolates selected is shown in Tables 17-19. In some instances isolates representing a variable were lost during storage and none was available for use. A total of 109 isolates was tested. All isolates tested were neutralized and confirmed as IPN virus.

#### Electron Microscopy

Infectious pancreatic necrosis virus samples were examined by electron microscopy to determine if any significant morphological differences between the ATCC stock virus (VR299) and a natural virus isolate could be observed. No viral aggregates could be seen in either sample and no apparent differences were detected. The diameters of the stock virus (Fig. 2) and a natural virus (Fig. 3) were measured as 63 and 68 nm respectively. Both diameters are in the range reported for IPN virus. The inability to detect significant differences between stock and natural virus was based on no striking differences in size, shape or morphologic units visualized. No structures external to the capsid were seen. Both nucleocapsids appeared to be hexagonal in shape.

Table 17. Natural Virus Isolates Selected for Identification from Isolations made during Studies of Salt, Eluent and Reconcentration Variables at Site B<sub>a</sub>

<u>Field Trial</u>	<u>Variables</u>						<u>Total Selected</u>
	Salt Enhancement		Eluent		With 2-Phase	Without 2-Phase	
	<u>With MgCl<sub>2</sub></u>	<u>Without MgCl<sub>2</sub></u>	<u>Beef Extract</u>	<u>Nutrient Broth</u>	<u>Separation</u>	<u>Separation</u>	
May 1976	-	0/10	0/ 7	0/ 3	0/10	0	0
June 1976	14/78	14/50	9/40	19/88	15/39	13/89	28
July 1976	51/80	22/56	38/60	35/76	40/80	33/56	73
Aug 1976	0/22	3/19	2/18	1/23	3/29	0/12	3
Sept 1976	2/18	3/23	3/22	2/19	3/29	3/12	5

<sup>a</sup>Number isolates selected/Number isolations made .

Table 18. Natural Virus Isolates Selected for Identification from Isolations made during Studies of Host Cell and Isolation Procedure Variables at Site B

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<u>Field Trial</u>	<u>Variables</u>				<u>Total Selected</u>
	<u>Host Cell</u>		<u>Isolation Procedure</u>		
	<u>CHSE</u>	<u>RTG-2</u>	<u>CPE</u>	<u>PFU</u>	
May 1976	0/8	0/2	0/7	0/3	0
June 1976	24/101	4/27	21/78	7/50	28
July 1973	29/61	44/75	68/100	5/36	73
Aug 1976	2/20	1/21	3/20	0/21	3
Sept 1976	5/9	0	5/5	0/4	5

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Number isolates selected/Number isolations made

Table 19. Natural Virus Isolates Selected for Identification from Isolations made during Studies of Virus Recovered from Individual Filters at Site B

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<u>Field Trial</u>	<u>Filter</u>				<u>Total Selected</u>
	<u>N39</u>	<u>E39</u>	<u>Cox</u>	<u>K27</u>	
May 1976	0/1	0/5	0/4	-	0
June 1976	8/37	11/40	-	9/51	28
July 1976	32/42	24/47	-	17/47	73
Aug 1976	1/8	2/18	-	0/15	3
Sept 1976	3/3	2/8	-	0	5

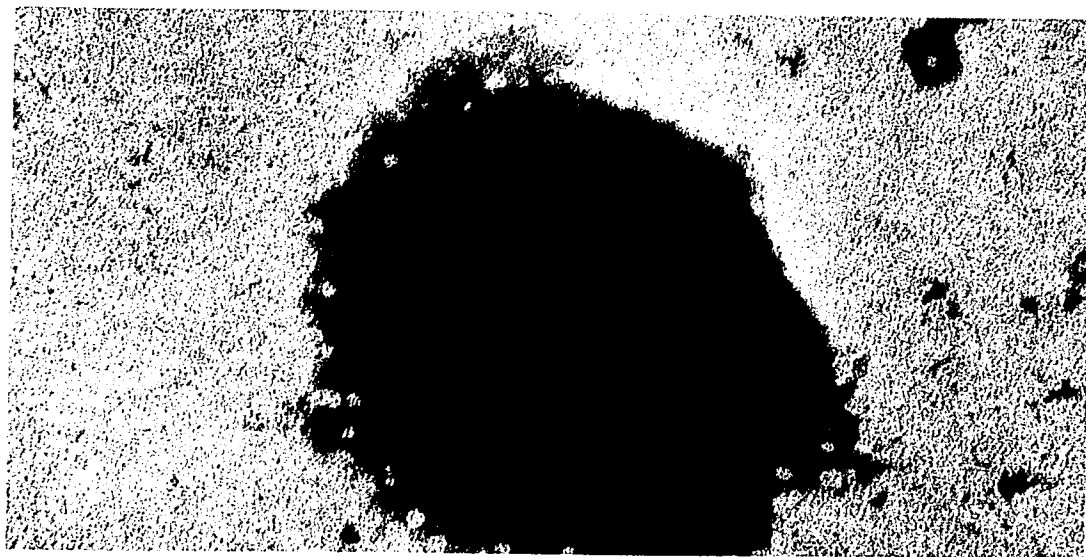
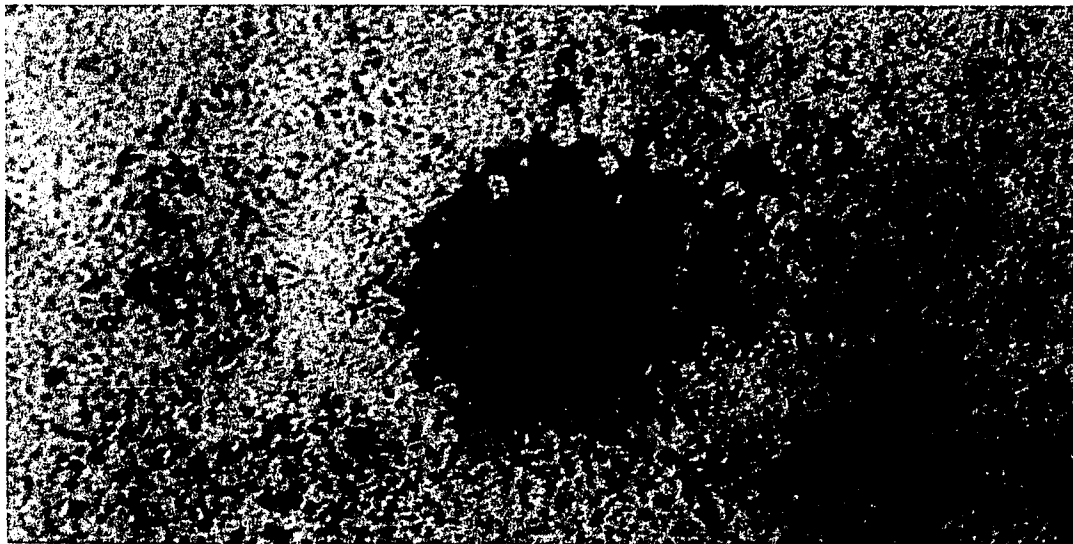
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Number isolates selected/Number isolations made

Figure 2. Electron Micrograph of Negatively Stained  
Infectious Pancreatic Necrosis Virus  
American Type Culture Collection Strain  
VR299. X 205,000.

Figure 3. Electron Micrograph of Negatively Stained  
Natural Infectious Pancreatic Necrosis  
Virus. X 205,000.





## DISCUSSION

Epizootics in fish hatchery populations caused by bacteria or viruses can have serious economic impact. Commercial aquaculture enterprises involved in the supply of fish for marketplace purchase or federal and state agencies involved in stocking of natural waters suffer severe economic losses when an epizootic occurs among fish stocks. Infectious pancreatic necrosis is a disease that can occur in epizootics. Infectious pancreatic necrosis virus has been isolated directly from hatchery waters only during outbreaks. The concentration of virus at these times apparently is large enough for detection from unconcentrated samples, due to the large number of infected and moribund fish continually shedding virus. Isolation of IPN virus from water before or after an outbreak when the virus is present in smaller amounts is dependent on an ability to concentrate small numbers of virus particles from large volumes of water. The development of a system for the detection and recovery of IPN virus represents a significant step toward ways and means of controlling IPN. Use of this system in field trials at the National Fish Hatchery at Berlin, New Hampshire, showed that IPN virus present in hatchery waters could be detected in advance of the outbreak of an epizootic in hatchery brook trout.

Field trials were conducted in October 1975 and monthly from May through September 1976. The first virus

isolations were made during the May trials. A large increase in the number of viral isolations occurred in June immediately before the recognition of an outbreak of IPN was made by hatchery personnel. The outbreak continued through July when the maximum number of isolations were made. More than 30% (approximately 82,000) of the fingerling brook trout population were lost during the epizootic. The number of virus recoveries declined rapidly after the July tests, but virus was still found in hatchery waters in August and September, more than a month after the epizootic had ceased. Direct isolation of natural virus from unconcentrated hatchery water samples was possible only during the height of the outbreak in July.

The field trials offered an opportunity for a more critical evaluation of the merits of some of the procedures developed with test virus but untried with natural virus under natural conditions. The key questions involved decisions on the usefulness of salt for enhancement of virus adsorption, the value of different adsorbent filters, choice of eluent, sample reconcentration, relative suitabilities for virus recoveries of CHSE versus RTG-2 cell cultures, and whether a plaquing or a CPE method is optimal for recovery of virus.

No great advantage for the use of  $MgCl_2$  was found with the model system in studies in which 190 l samples of clean water were tested. Results in the field trials indicated that  $MgCl_2$  enhanced slightly the recovery of

natural virus. Sobsey et al. (1973) reported that use of  $MgCl_2$  was most likely to be of value when enterovirus recoveries from very turbid water were considered. Since the turbidity of the Berlin hatchery waters was not excessive no opportunity for demonstration of  $MgCl_2$  enhancement of virus recovery was presented. The slight enhancement of virus recovery found in relatively clean water suggested routine use of  $MgCl_2$  should be considered unless contraindicated by logistic factors.

Selection of nylon (N39) filters for sample clarification and viscose (E39) and fiberglass (K27) for virus adsorption provided an effective recovery system. Developmental work indicated that a nylon (N39) filter allowed passage of 100% of the virus if no suspended solids were present in the water. Suspended solids did interfere to a minor degree with passage of the virus in model system studies but with elution of the clarifying filter, an average of 80% of the adsorbed virus was recovered. Isolations from the clarifying filters as a result of the use of this procedure during field trials further indicated the necessity to continue elution of clarifying filters.

The recovery of natural virus from viscose (E39) and fiberglass (K27) filters showed both filters were effective adsorbers under the field conditions encountered. Viscose (E39) filters experienced some breakthrough of test virus in the model system and this was also found in the field trials. Many natural virus recoveries from fiberglass

filters downstream from the viscose (E39) filters indicated the occurrence of natural virus breakthrough. The reason for the breakthrough was not investigated but it could be speculated that virus receptor sites on viscose (E39) filters were occupied or interfered with by the presence of MCC in the water sampled. The most likely source of MCC was fish derived products such as feces or slime. The breakthrough showed the importance to virus recovery of the practice of using viscose (E39) and fiberglass (K27) filters in series.

The effectiveness of cyclic elution using 5X nutrient broth for elution of virus from filter surfaces was attributed to the influence in part of a proteinaceous solution with a greater affinity than virus for virus adsorptive sites on filters, and in part to an alkaline pH causing reversal of the initial electrostatic attractive force binding virus to a filter surface. The greater number of recoveries made with 5X nutrient broth, compared to those made following elution with 3% beef extract, was attributed to the former eluent's greater concentration of proteinaceous substances resulting in a more effective displacement of virus.

The effectiveness of the aqueous polymer two-phase system A for recovery of test virus in model system studies was confirmed by the results of the field trials. Reconcentration of samples was beneficial in a majority of the trials. Results of the May and September trials

suggested reconcentration would be especially effective where minimal virus numbers were likely to be encountered.

The greater number of natural virus recoveries made with CHSE monolayers suggested a greater sensitivity than RTG-2 monolayers for the isolation of IPN virus. The use of liquid overlays and reliance on CPE endpoints for recognition of the presence of virus was preferable to PFU endpoints. If a single recovery method is to be used to monitor the virus content of hatchery waters, CPE procedures combined with CHSE cultures represent the method of choice.

All isolations of natural virus demonstrated the same distinctive CPE or plaque morphology typical of IPN virus. It was necessary to provide further evidence that these isolates were indeed IPN virus and serum neutralization tests with 7-strain polyvalent IPN virus antiserum were performed. Testing of representative isolates from each variable evaluated confirmed the identification of isolates as IPN virus.

The optimal virus concentration method for recovery of IPN virus from fish hatchery waters consisted of a nylon (N39) clarifying filter, lowering the pH to 6.0 and the addition of  $MgCl_2$  to a final concentration of 0.05 M, and adsorption to viscose (E39) and fiberglass (K27) filters arranged in series. Virus recovery was best accomplished by the use of cyclic elution with 5X nutrient broth, pH 9.0, followed by reconcentration of the eluate using aqueous polymer two-phase system A. The most effective recovery of

natural virus from test samples was made using CPE endpoints on CHSE inoculated cultures. The recovery method described optimally was calculated to have been capable of detecting IPN virus in hatchery waters in concentrations as low as 1 infective particle per 166 l.

Study results substantiate the usefulness of the system described as a means for detecting and enumerating IPN virus. This capability is of potential value as an early warning of the presence of a dangerous fish pathogen and offers an opportunity to initiate measures appropriate for the prevention and control of IPN epizootics.

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