AN ABSTRACT OF THE THESIS OF

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Title: VIRUSES INFECTING SALMONID FISHES FROM OREGON

A. THE OCCURRENCE AND DISTRIBUTION OF INFECTIOUS PANCREATIC NECROSIS VIRUS

B. THE DEVELOPMENT OF AN ATTENUATED STRAIN OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS FOR THE IMMUNIZATION OF SALMONIDS

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Abstract approved: _____
Dr. J. L. Fryer

The occurrence and distribution of fish viruses in Oregon were determined by the examination of anadromous and fresh water salmonids. Infectious pancreatic necrosis virus (IPNV) was isolated and identified from asymptomatic adult coho (Oncorhynchus kisutch) and chinook (O. tshawytscha) salmon returning to the Columbia River system. Infectious pancreatic necrosis virus was also identified as the causative agent of severe losses of eastern brook trout juveniles (Salvelinus fontinalis) at the Fall River trout hatchery in central Oregon. This was the first documented IPNV epizootic in fish from Oregon. A high incidence of IPNV was detected in eastern brook trout populations from central Oregon indicating that this virus poses

a threat to the rearing of that species in Oregon.

An attenuated strain of infectious hematopoietic necrosis virus (IHNV) was developed for use as a water-borne vaccine for the control of IHNV disease. This water-brone vaccine is easily administered as an immunizing agent by direct addition to the water containing fish. The attenuated IHNV water-borne vaccine strain was determined to be one hundred three times less virulent than the wild type strain from which it was derived. This vaccine was proven to be efficacious against high levels of both water route and injected challenges of virulent wild type virus in kokanee (O. nerka) and sockeye salmon (O. nerka).

Other parameters for the development of an effective vaccine preparation were also tested. An optimal exposure period to the immunizing virus of 48 hours was determined as were the minimum immunizing dose for sockeye and chinook salmon. The attenuated IHNV water-borne vaccine produced immunity with a duration of at least 110 days and was shown to provide protection to sockeye salmon challenged with four isolates of virulent IHNV from different geographic locations. Another method of immunization, vacuum infiltration, was tested using a second strain of attenuated IHNV as an immunizing agent. Even though this method of immunization was effective in eliciting a protective immune response in chinook and kokanee salmon, it did not produce the level of protection provided by the attenuated IHNV water-borne vaccine.

Viruses Infecting Salmonid Fishes from Oregon

A. The Occurrence and Distribution of Infectious Pancreatic Necrosis Virus

B. The Development of an Attenuated Strain of Infectious Hematopoietic Necrosis Virus (IHNV) for the Immunization of Salmonids

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INTRODUCTION

Certain viruses infecting salmonid fishes pose a threat to the continued culture of these animals in Oregon. The increased number of viral isolations, in recent years, has created the need to establish the overall significance of this problem. Determination of the occurrence and distribution of fish viruses, as well as development of methods to prevent and control the fish diseases they cause, are essential in maintaining healthy populations of salmonids.

After the isolation of the Oregon sockeye virus (OSV) in 1958, no fish viruses were observed from any population of salmonids in Oregon until the recovery and identification of infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV) in 1971 (McMichael, 1974). Because no methods were available for the prevention and control of IHNV, a study was initiated in 1972 designed to develop attenuated strains of this virus for use as immunizing agents in salmonids. As a direct result of that work, the following investigatigations were undertaken to examine more extensively viruses infecting salmonid fishes from Oregon.

The objectives of this study were to determine the occurrence and distribution of fish viruses and to test an attenuated strain of IHNV developed by McMichael (1974) for the immunization of salmonids. Populations of anadromous and fresh water salmonids from central Oregon were to be examined for the presence of viruses. In addition, in vivo experiments to develop an efficacious, easily administered water-borne vaccine against IHNV were conducted. The degree of attenuation of the developed IHNV water-borne vaccine strain was to be determined by virulence comparisons with the wild type strain from which the modified virus was derived. Experiments included the comparison of efficacy of the vaccine to both water route and injected challenges in two species of salmonids. Immunization doses, as well as exposure periods to the water-borne vaccine, also were to be determined. Other parameters studied were the duration of immunity and the ability of the vaccine preparato confer protection to challenges with virulent IHNV strains from different geographic locations. An alternate method of vaccination (direct injection) using a second strain of attenuated IHNV was also explored.

LITERATURE REVIEW

Infectious Pancreatic Necrosis Virus (IPNV)

A fish disease termed acute catarrhal enteritis was described in salmonids by M'Gonigle (1941) in 1940. This disease was renamed infectious pancreatic necrosis (IPN) in 1953 (Snieszko and Wolf, 1958). Infectious pancreatic necrosis virus (IPNV) was first reported in the western United States by Parisot et al. (1963). MacKelvie and Artsob (1969) documented the presence of the disease in nine locations in the Canadian Maritime Provinces. Wolf and Pettijohn (1970) described the isolation of IPNV from coho salmon (Oncorhynchus kisutch) at the Lamar, Pennsylvania, National Fish Hatchery. McMichael (1973) isolated IPNV from coho salmon at the Bonneville salmon hatchery in Oregon during 1971. Both recoveries of IPNV were from fish that were not suffering any virus related deaths. Yamamoto (1974) reported the occurrence of the virus at a hatchery in Alberta, Canada. According to Wolf (1972), Denmark, Sweden, France, Italy, the United Kingdom and Japan have all had IPNV outbreaks.

The infectious nature of the disease was first demonstrated experimentally by Snieszko et al. (1959) and Wood et al. (1955). A viral etiology of the disease was first reported by Wolf et al. (1960). Wolf et al. (1961) and Wolf and Quimby (1971) experimentally

demonstrated that IPNV can cause an acute disease associated with high levels of mortality in young salmonids, however, in older fish, Snieszko et al. (1959) determined there is a reduction in mortalities. The clinical symptoms of IPNV have been described by Wolf (1966) and the histopathology has been determined by Yasutake (1970).

The classification of IPNV has not been definitely resolved. Cerini and Walsberger (1965) described IPNV as a picornavirus with 12 capsomeres and a diameter of 18-29 nm. The first electron micrographs are credited to Moss and Gravell (1969) who described the virus as a reovirus with 92 capsomeres and a diameter of 55-65 nm. There has also been some disagreement concerning the nature of the nucleic acid of this virus. Argot (1969) presented evidence that the RNA of IPNV is double stranded. Nicholson (1971) suggested that the RNA is probably single stranded, and Kelly and Loh (1972) concluded from cytochemical and autoradiographic studies that IPN virus RNA is single stranded. These authors suggest that the virus does not fit in either the reovirus or picornavirus taxonomic groups. Lientz and Springer (1971) demonstrated that within this virus group, as many as seven serologic types of IPNV may exist as determined by neutralization experiments with polyvalent antiserum.

Infectious Hematopoietic Necrosis Virus (IHNV)

Infectious hematopoietic necrosis virus (IHNV) has been isolated

from salmonid fishes in the United States on several occasions. Rucker et al. (1953) reported epizootics among the genus O. nerka in the state of Washington and suggested a viral etiology. A filterable agent was demonstrated and it was proposed that it be called the sockeye salmon virus (Watson, 1954). Parisot et al. (1965) changed the name to the Columbia River sockeye disease. Later, a similar disease of sockeye salmon, detected in Oregon by Fryer (1964) was termed the Oregon sockeye virus (OSV). The status of the sockeye virus has been reviewed by Guenther et al. (1959); Amend et al. (1969); Wingfield et al. (1969) and Amend and Chambers (1970). A virus-like disease of chinook salmon (O. tshawytscha) in the Sacramento River, California was described by Ross et al. (1960) and Parisot (1962). This virus was named the Sacramento River chinook disease (SRCD) virus. Recent isolations of IHNV have been reported in Oregon (McMichael, 1973) and in Washington (Amend and Wood, 1972). Wolf et al. (1973) documented the occurrence of IHNV in eastern North America.

The physical and biochemical properties of the sockeye and chinook viruses were compared and found to be similar (Wingfield et al., 1969; Wingfield and Chan, 1970; Amend and Chambers, 1970). A virus recovered from rainbow trout (Salmo gairdneri) by Amend et al. (1969) was morphologically and biochemically compared to the OSV and SRCD virus isolates (Darlington et al., 1972; Amend and

Chambers, 1970). The results of these studies showed no differences between the three isolates. Using cross neutralization experiments to determine the antigenic relationships among the three isolates, McCain et al. (1971) concluded that they were closely related. It has now been generally accepted that the three viruses are the same or closely related and are referred to as IHNV (Amend et al., 1969).

Clinical signs of the disease have been described by Amend (1970). Histopathological examination of infected fish revealed that the spleen and kidney were the foci of infection (Yasutake et al., 1965; Yasutake and Amend, 1972).

The pathogenesis of IHNV disease has been described by Yasutake and Amend (1972) and the pathophysiology of the disease in rainbow trout was determined by Amend and Smith (1974). McAllister and Pilcher (1974) demonstrated auto-interference in IHNV replication and a further characterization of the virus was reported by McAllister et al. (1974). The physiochemical properties of IHNV RNA were determined by McCain et al. (1974).

Detection of Fish Viruses

Methods for the detection of fish viruses have been reported by Amend (1970) for IPNV and by Amend and Wedemeyer (1970) for IHNV. McMichael (1974) also described methods used for isolating and identifying fish viruses. More recently, the Fish Health Section

of The American Fisheries Society has published suggested procedures for the detection of certain fish diseases (1975).

The Immune Response of Salmonids to Fish Viruses

No methods have been developed for the prevention of fish viral diseases and only one method has been reported for controlling them. Amend (1970) demonstrated that IHNV disease could be controlled by elevating the water temperature. Wolf (1963) demonstrated that trout respond immunologically after contact with a fish virus and Wolf and Quimby (1969) showed that rainbow trout could produce high titers of neutralizing antibodies after injection with virulent IPNV. According to Dorson (1972), rainbow trout produced neutralizing antibodies to the bacteriophage (FH_5). Jorgensen (1971) showed that rainbow trout formed neutralizing antibodies to injected viral hemorrhagic septicemia (VHS) virus. The response was slow and it took 18 months to detect neutralizing antibodies. McMichael (1974) described two attenuated IHNV strains that produced immunity in O. nerka. Amend (1974) reported that rainbow trout produced antibodies after injection with IHNV and passively immunized fish were protected with these antibodies.

Fish virology is a relatively new scientific field that is expanding rapidly. Two excellent review articles on fish virology have been written by Wolf (1966; 1972).

MATERIALS AND METHODS

Cell Lines

Two salmonid cell lines were used for viral isolation, propagation, and infectivity assays. These were cell line CHSE-214, derived in this laboratory from chinook salmon (Oncorhynchus tshawytscha) embryos in 1964 and cell line STE-137, derived from steelhead trout (Salmo gairdneri) embryos in 1963 (Fryer et al., 1965). The CHSE-214 cell line was used for viral isolation from visceral, whole fish and ovarian fluid samples and also for all plaque assays, neutralization experiments and for virus production for both specific antisera production and in vivo vaccine studies. The STE-137 cell line was used solely for viral isolation from the various sample types.

Maintenance and Propagation of Stock Salmonid Cell Cultures

Stock cell cultures were propagated at 18 C in 32 oz prescription bottles stoppered with latex stoppers (West Co.). Subcultures were made every ten to twenty days as described by McCain (1970). The growth medium was changed only if the pH of the medium became very acidic.

Sterility checks were performed by periodically inoculating one

milliliter (ml) of the cell culture into Mycoplasma Broth (Pfizer and Co.) and into Fluid Thioglycollate Medium (BBL). These sterility checks were incubated at 37 C and 18 C for two weeks.

Media Used for Cell Culture Propagation

The two salmonid cell cultures were maintained in Eagle's minimal essential medium (MEM) (Eagle, 1959). This medium was prepared by adding MEM components (Flow Laboratories, Inc.) to Hank's balanced salt solution (HBSS) or by adding double distilled water to powdered cell culture media (Flow Laboratories, Inc.) containing Earle's balanced salts (EBSS). Both types of cell culture media contained 10% fetal bovine serum (Flow Laboratories, Inc.) and streptomycin (100 mg/ml), and penicillin (100 IU/ml). This cell culture medium will be referred to as MEM-10%.

Tissue culture media used for propagation of viruses and diagnostic procedures contained only 5% fetal bovine serum. This medium will be referred to as MEM-5%.

Viruses

All viruses used throughout this study were propagated on CHSE-214 and STE-137 cell lines at 18 C, except the infectious hematopoietic necrosis virus (IHNV)-71-Attenuated-injectable viral

vaccine strain which was propagated at 9. 5 C. Stocks of all viruses have been pre served by freezing at -60 C.

Standard Reference Viruses

Wild Type Infectious Hematopoietic Necrosis Virus

The reference strain of IHNV (wild type IHNV) was isolated and identified by McMichael (1973) in 1971 from infected rainbow trout (S. gairdneri) in Nan-Scott Lake. This virus was used in antiserum production, as a challenge virus in immunization experiments and as a control virus in serum neutralization studies.

Infectious Pancreatic Necrosis Virus (IPNV)

The reference strain of IPNV (CTT-IPNV) was isolated and identified by McMichael in 1971 from native cutthroat trout (S. clarki) in an outlet creek of Nan-Scott Lake. This virus was used for antiserum production and as a control virus in serum neutralization experiments.

Other Strains of Virulent Infectious Hematopoietic Necrosis Virus

Other isolates of IHNV were also used as challenge viruses in these studies. Table 1 lists the name and source of these viruses.

Table 1. Sources of other isolates of virulent IHNV used as challenge viruses in these studies.

Virus	Source
Oregon sockeye salmon virus (OSV)	Isolated from sockeye salmon (Oncorhynchus nerka) by J. L. Fryer, 1958.
Washington Cedar River (IHNV)	Isolated from sockeye salmon kindly furnished by Dr. D. F. Amend, Western Fish Disease Laboratory, Seattle, Wash.
Sacramento River chinook virus (SRCV)	Isolated from chinook salmon kindly furnished by Dr. W. H. Wingfield, California Department of Fish and Game, Rancho Cordova, California

Attenuated Infectious Hematopoietic Necrosis Virus Vaccine Strains

Two strains of attenuated IHNV to undergo extensive testing were developed by McMichael in 1971 (1974). They will be referred to as IHNV-71-Attenuated-water-borne viral vaccine (attenuated IHNV water-borne vaccine) and IHNV-71-Attenuated-injectable viral vaccine (attenuated IHNV injectable vaccine). These viruses were derived by multiple passage of the wild type IHNV strain on the STE-137 cell line at 18 C and 9.5 C respectively.

Propagation of Salmonid Viruses

All viruses, except the attenuated IHNV injectable vaccine strain, were propagated in the same manner. Glass tubes or vials containing the frozen stock preparations of the viruses were removed from the -60 C freezer and thawed at 18 C. The tissue culture medium was removed from near confluently or confluently monolayered C HSE-214 cells in a 32 oz prescription bottle containing MEM-10% and was replaced with 49 ml of MEM-5%. A portion of the thawed virus preparation was diluted 1:100 in MEM-5%, and one ml was added to the cell culture which had received 49 ml of MEM-5%. This resulted in the original viral stocks being diluted 1:5000. The inoculated cell cultures were incubated at 18 C for 48 to 96 hours except the attenuated IHNV injectable vaccine strain, which was incubated at 9.5 C.

Viruses were harvested after the cell culture showed typical cytopathic effects (CPE). Virus-containing tissue culture medium was removed and centrifuged at 2,200 x g for 20 minutes to remove cell debris. The supernatant, containing the virus particles was dispensed in one or two ml portions into sterile deep skirted specimen vials with screw caps and frozen at -60 C. Stock preparations were sterility tested in fluid thioglycollate medium and mycoplasma broth as previously described. To account for loss of infectivity

due to freezing, the titer of the stock preparation was determined after the viruses had been frozen at -60 C for ten to fourteen days.

The Monolayer Plaque Assay Technique

Infectivity titers for all strains of IPNV and IHNV were determined by the monolayer plaque assay technique. Two oz prescription bottles were seeded with five ml of a CHSE-214 cell suspension seven to ten days before use. Four days after seeding, the cell medium was replaced with fresh medium. Before use, all bottles were examined microscopically to insure that the monolayers were complete. Ten-fold dilutions of the virus preparation to be assayed were made in MEM-5%. Cell culture medium was removed from each monolayered prescription bottle and replaced with 0.3 ml of a viral dilution. Three bottles were inoculated with each viral dilution. This viral inoculum was allowed to adsorb onto the cell sheet for one hour at 18 C, and even distribution was insured by gently rocking the bottles every 15 minutes.

At the conclusion of the one hour adsorption period, a nutrient agar overlay was added. This overlay was prepared by mixing equal volumes of 2X MEM-5% heated to 45 C and melted 1.5% Ionagar (Colab Laboratories, Inc.) in double distilled water. This overlay solution was added to each assay bottle in five ml aliquots with a Cornwall syringe. After the overlay solidified, the bottles were

inverted and incubated at 18 C.

After viral plaques formed (24 to 48 hours for IPNV, 72 to 96 hours for IHNV), a second overlay was added. This overlay was identical to the first, except neutral red, at a final concentration of 50 μg/ml in double distilled water, was added in place of serum to the overlay. After the overlay solidified, the bottles were inverted and incubated at 18 C in the dark (Green et al., 1959).

Twenty-four and 48 hours after addition of the second overlay, bottles containing 20 to 200 plaques were counted. The plaques from each of the three bottles at each dilution were averaged, and from this average, the titer in plaque forming units (pfu) per ml was calculated.

Rabbit Anti-Viral Immune Serum Preparation

Rabbit antiserum against CTT-IPNV and wild type IHNV were prepared for identification of viral agents and for confirmation of viral infection in in vivo experiments. The following methods were used to prepare viral antigen. Stock virus was propagated on CHSE-214 and harvested as previously mentioned. The viral suspension then was centrifuged at 2, 200 x g for 20 minutes after which time the supernatant was collected and centrifuged at 55, 000 x g for 60 minutes. The resulting viral pellets were resuspended to 1/100 of their original volume in MEM-5% and this preparation was stored

at 4 C for immediate use or -60 C for use at a later time.

Prior to immunication, each rabbit was bled from the marginal ear vein. The serum was harvested and tested for anti-wild type IHNV and anti-CTT-IPNV antibodies by the serum neutralization procedure.

Each rabbit was immunized according to the following schedule. First, one ml of the viral preparation was injected intravenously. At the same time, the animal received two one ml intramuscular (im) injections, one in each flank. The preparations for the im injections consisted of a thoroughly emulsified sterile suspension containing equal volumes of the viral preparation and complete Freund's adjuvant (Difco). The emulsion was tested for uniformity by allowing it to stand at 4 C for 20 minutes. Two and four weeks after the initial injection, the rabbits received two additional one ml im injections.

Six weeks after the initial injections, the rabbits were bled from the marginal ear vein. The blood was collected in sterile 50 ml centrifuge tubes and incubated at 4 C for clot separation. The serum was collected, sterilized by passing through a 0.45 μ membrane filter in a Millipore Filtration system, dispensed in two ml portions into glass specimen vials with screw caps and stored at -60 C.

The Occurrence and Distribution of Fish Viruses

Sampling Locations

Stocks of fish to be examined for viral agents were determined as follows: (1) Hatchery locations for the examination of returning adult anadromous salmonids and their offspring were determined by the Fish Commission of Oregon. (2) Hatchery and field locations for the examination of landlocked salmonids in central Oregon were determined by the Oregon Wildlife Commission. (3) Fish were examined by request from a number of locations experiencing abnormal mortalities in their stocks.

Types of Samples Collected

Ovarian fluid, visceral samples containing kidney, spleen and liver, and whole fry samples were the types of samples examined.

Collection of Samples for Examination

In most cases when the size of the population was known, the sample size consisted of 60 animals which allowed for the detection of a 5% incidence level within a 95% confidence interval (Amend and Wedemeyer, 1970).

When it was not feasible to sample at a 5% incidence level, as many animals as possible were examined. The types of samples

collected were the same regardless of the size of the population being sampled.

Both ovarian fluid and viscera were sampled from returning adult female anadromous fish. Two ml of ovarian fluid were collected from egg containers after five females had been spawned into that container. Thus, from a sample population of 60 fish, twelve ovarian fluid samples were collected. Visceral samples consisted of 0.1 g portions of kidney, spleen and liver from each of 60 fish. All visceral samples from each of five fish were pooled, resulting in 12 visceral samples from a sample population of 60 fish. Only visceral samples were collected from fish at times other than spawning periods. Yolk sac fry and juvenile fish less than two inches in length were examined as whole fish samples. All samples were placed into sterile screw capped tubes on ice and transported back to the laboratory where they were stored at 4 C until examination. All samples were examined as soon as possible or within 96 hours after they were collected, and, except for rare cases, the samples were never frozen.

In most cases, blood samples were collected by severing the caudal artery and collecting the blood in sterile screw capped tubes. Blood was collected from very small fish by severing the caudal artery and collecting it in 50 microliter capillary tubes. A heart puncture method was used to obtain blood from adult salmon during their ocean phase. All blood samples were placed on ice, transported

back to the laboratory and stored at 4 C until the serum phase had separated.

Examination of Samples

All samples were examined using two cell lines, CHSE-214 and STE-137. Forty-eight hours prior to use, the cells were seeded onto flat-bottomed plastic tissue culture microplates sealed with pressure sensitive films (Cooke Engineering Co.). Each sample was placed into six seeded wells of a microplate and incubated at 18 C, and were examined microscopically at least once every 24 hours for the appearance of CPE.

Ovarian Fluid Samples. Ovarian fluid samples were diluted 1:4 in MEM-5% and filtered directly onto the two cell cultures through a 0.45 μ membrane filter in a Swinny Filter holder (Millipore Corp.). A sample of 0.1 ml was added to 0.1 ml of MEM-5% in each of six wells of the two microplates.

Visceral Samples. Twenty ml of buffered (pH 7) HBSS was added to each five fish pool of visceral samples and homogenized at 23,000 rpm for two minutes with a VirTis Model 23 Macro Homogenizer. The resulting homogenate was centrifuged at 3,000 x g at 4 C for 15 minutes. The supernatant was diluted 1:4 in an antibiotic solution contining 10,000 IU/ml of penicillin, 10,000 μg/ml streptomycin sulfate, and 10,000 IU/ml nystatin dissolved in HBSS or EBSS

(Amend and Pietsch, 1972; McMichael, 1973). The antibiotic-sample mixture was stirred and incubated at 22 C for two to six hours.

The mixture then was centrifuged at 7, 700 x g for 20 minutes to clear the solution of cellular debris. The supernatant from this centrifugation was passed through a Swinny Filter holder containing a 0. 45 μ membrane, and the filtrate was inoculated directly onto the cell cultures. Six wells of two tissue culture microplates were inoculated with 0. 2 ml of each sample.

Whole Fish Samples. Fish less than two inches in length were examined as whole fish samples. Several fish were pooled together in various numbers depending upon their size and examined by the same methods used for visceral samples.

Identification of Viral Agents from Collected Samples

Viral agents were isolated and identified from visceral whole fish and ovarian fluid samples by the following methods. When CPE was observed in cell cultures seeded with a sample, the tissue culture fluid was withdrawn from each infected well of a sample with a one ml tuberculin syringe. The tissue culture fluid, suspected of containing virus, was diluted 1:100 in MEM-5% and reinoculated onto newly seeded cells of the same cell line from which the sample was withdrawn. If CPE was observed after the first subculture, the

sample was subcultured again in the identical manner. This process was repeated until the sample had reached an overall dilution of at least 1×10^{-6}. This method of serial subculturing served to dilute toxic factors from the sample which might cause cellular degeneration similar to viral CPE.

If the sample still showed CPE after an overall dilution scheme of 1×10^{-6}, it was inoculated into a recently monolayered 32 oz prescription bottle culture of the original cell line from which it was isolated.

Final identification of the agent was made by neutralization experiments using specific rabbit antisera against known salmonid viruses.

Identification of Viral Agents
by Serum Neutralization

Serum neutralization experiments were used for the final identification of viral agents isolated from samples and also for the identification of IHNV from infected fish in base line kill curve experiments.

Tenfold dilutions of the isolated agent were made in MEM-5% and one ml of each dilution was mixed with one ml of rabbit anti-IPNV and anti-IHNV serum diluted 1:100 in MEM-5%. This mixture was incubated for 60 minutes in sterile screw capped tubes to allow the formation of antigen-antibody complexes. After the incubation

period, 0. 2 ml of this mixture was placed in each of four wells of a microplate containing a 48 hour culture of CHSE-214 cells. Sample dilutions of 0. 1 ml not incubated with the antisera were also placed in four wells of the same microplate as controls. Known virus controls and antiserum controls were also used.

The microplate was examined daily and positive identification was made by observing which specific antisera neutralized the sample CPE. The controls were carefully observed for the expected results.

Detection of Specific Neutralizing Antibodies in Blood Samples

At certain locations, blood samples were collected from fish at the same time they were being sampled for viral agents. The serum phase that was harvested from the blood sample was examined for the presence of specific anti-viral antibodies.

Serum harvested from blood samples was diluted 1:50, 1:100 and 1:200 in MEM-5% and filtered through a 0. 45 μ membrane filter in a Swinny Filter holder. Two ml of each serum dilution was added to two ml of the known virus suspension, resulting in a final concentration of 50 pfu in 0. 1 ml. The virus-serum mixtures were incubated at 18 C for 60 minutes. Each serum sample was tested for neutralizing effects against both IPNV and IHNV.

After the incubation period, 0. 2 ml of each mixture was placed

in four microplate wells seeded with cells 48 hours prior to use. Controls used were: (1) serum samples not incubated with virus, (2) known virus samples not incubated with serum, and (3) cells containing MEM-5%.

Plates were examined daily for neutralizing effects exhibited by the serum samples. Serum samples that neutralized the known viral CPE were regarded as positive for specific neutralizing antibody.

Development of a Viral Vaccine Against Infectious Hematopoietic Necrosis Virus

The following methods and viruses were tested for immunizing ability in sockeye and chinook salmon: (1) an attenuated IHNV waterborne vaccine strain and (2) a vacuum infiltration method of immunization using attenuated IHNV injectable vaccine as the immunizing agent. Efficacy of immunization was tested by challenging the vaccinated fish with virulent challenge viruses administered by an intraperitoneal (ip) injection or by water route exposure.

In all in vivo immunization experiments, three sets of controls were used. One group was neither immunized nor challenged and was used to determine the effect of normal experimental conditions on the fish. Since the vaccine consisted of a live modified virus, another group was immunized, but not challenged and these control

fish were used to determine whether the immunization procedure had an adverse effect on the animals. The third control consisted of animals that were not immunized, but did receive a challenge dose. This group determined the effectiveness of the challenge virus.

Experimental Animals

Two species of experimental animals were used for all in vivo work. The sources of these animals are listed in Table 2.

Table 2. Sources of experimental animals used in in vivo experiments.

Experimental animal	Source
Sockeye salmon - *Oncorhynchus nerka*	Environmental Protection Agency - Western Fish Toxicology Station Corvallis, Oregon
Kokanee salmon - *O. nerka*	Wizard Falls Hatchery Oregon Wildlife Commission Camp Sherman, Oregon
Fall chinook salmon - *O. tshawytscha*	Fish Commission of Oregon's Trask Hatchery, Tillamook, Oregon and Big Creek Hatchery, Astoria, Oregon

Fish ranged from 0.2 to 1.5 g in weight, and all groups of fish were examined for the presence of viral agents and specific anti-viral neutralizing antibodies prior to use in experiments.

All fish used were transported from hatcheries to the Oregon State University Fish Disease Laboratory (FDL), Corvallis, Oregon

in 30 gallon plastic cans containing 20 gallons of hatchery water. At the (FDL) the fish were maintained in 18 gallon rectangular fiberglass aquaria at 7, 9.5 and 12.2 C until experimental use. The water source was pathogen-free well water with an ambient temperature of 12 C. All fish were tempered to 12.2 C for 48 hours before being transferred for experimental use.

Experiments were conducted in closed system plastic aquaria containing 10 liters of dechlorinated, defluoridated water at 12.2 C. The temperature was maintained by holding the aquaria in constant temperature incubators.

Each aquarium contained 20 fish. During the course of all experiments, oxytetracycline HCl (Pfizer and Co., Inc.) was added to the water at a concentration of two parts per million to prevent bacterial infections in the experimental fish. Fish were fed twice a day with either Rangen Salmon Mash (Rangen Fish Foods) or Oregon Moist Pellets (OMP) (Hublou, 1963), depending on the size of the fish. Fish were held at constant low illumination during experimental periods.

Aquaria water was changed every 48 hours. Fish were transferred to different aquaria containing clean 12.2 C water. The used water was sterilized by boiling for 30 minutes and discarded. Aquaria were autoclaved and cleaned before use.

Establishment of Base Line Kill Curves

Base line kill curves were determined for both the attenuated IHNV water-borne vaccine strain and for wild type IHNV. Sockeye salmon weighing 0.3 g were injected with 0.015 ml of virus diluted in HBSS ranging from undiluted (10^0) to 10^{-7}. Twenty fish were injected ip with each viral dilution. Controls were injected with 0.015 ml of HBSS.

Dead fish were collected four times daily, recorded, and frozen at -20 C. All animals which died were examined for the presence of viral agents and final identification was made by observing the appearance of characteristic CPE. Each experiment was terminated 21 days after injection, at which time the LD_{50} of both the attenuated IHNV water-borne vaccine strain and the wild type IHNV was calculated by the method of Reed and Muench (1938). After the LD_{50} was calculated for each virus, it was correlated to the number of plaque forming units in each LD_{50} dose (pfu/LD_{50} ratio). The mean day to death (MDD) for each group was calculated using the equation

$$MDD = \frac{\Sigma \text{ (number of deaths) (day of death)}}{\text{total number of dead fish}}$$

Immunization with IHNV-71-Attenuated-Water-Borne Viral Vaccine

Experimental fish were exposed either to undiluted or diluted tissue

culture fluid containing the attenuated virus as an immunizing dose. Different levels of the immunizing virus in MEM-5% were administered according to the experiment being conducted. The exposure period to the water-borne immunizing agent was also varied, although a 48 hour exposure period was used most often. Fish were immunized by adding infected tissue culture fluid directly to aquaria water containing twenty fish.

Vacuum Infiltration Immunization with IHNV-71-Attenuated-Injectable Viral Vaccine

McMichael (1974) demonstrated that the attenuated IHNV injectable vaccine was an effective immunizing agent when administered by an ip injection. This virus was experimentally administered by a method known as vacuum infiltration, a delivery system for the immunization of fish developed by Wildlife Vaccines, Inc., Wheatridge, Colorado. For this type of immunization, 20 fish were placed in a five l plastic vacuum desiccator containing one l of dechlorinated water. The vaccine preparation, consisting of undiluted tissue culture fluid, was added to the one l of dechlorinated water. Vaccine preparations tested contained 7,700, 77,000 and 385,000 pfu of attenuated virus per ml of water in the desiccator. The atmosphere in the desiccator was brought down to 240 mm of mercury with a 1/6 horsepower electric vacuum pump, and maintained at that level for

30 seconds at which time it was immediately released to normal atmospheric pressure. This process was repeated three times. After the vacuum infiltrations, the twenty fish were removed from the desiccator and placed in aquaria held at 12.2 C.

Challenges to Test the Efficacy of Immunization

Efficacy of immunization was tested by challenge experiments with immunized fish after an immunization period, the period between immunization and challenge. In most experiments conducted, the immunization period was 21 or 25 days. However, in two experiments, an immunization period of 46 days was used while in another, 110 days was employed.

Injected Intraperitoneal (IP) Challenge

Various LD_{50} doses of challenge virus were administered ip. The LD_{50} dose for the wild type IHNV was calculated from data obtained in the base line kill curve experiment. All injected challenge levels of the wild type IHNV were determined as a multiple of the LD_{50} dose and calculated from the pfu/LD_{50} ratio.

Challenge doses containing viral dilutions in EBSS or HBSS were administered in 0.015 ml or 0.02 ml amounts. Controls were injected with 0.015 ml or 0.02 ml of HBSS or EBSS. Challenge levels of wild type IHNV consisted of 46 x LD_{50}, 48 x LD_{50}, 460 x LD_{50},

4,600 x LD_{50} and 46,000 x LD_{50}.

The SRCV virus was also used in two experiments as an injected challenge virus. Since the LD_{50} for SRCV was not determined, an arbitrary dose of 180 pfu of SRCV injected in 0.02 ml EBSS was used as a challenge. Controls were injected with 0.02 ml of EBSS.

Water Route Challenge

Water route challenges were developed to simulate a more natural condition. Undiluted, infected tissue culture fluid was used as the challenge dose and administered by direct addition to 10 l of aquaria water for an exposure period of 48 hours. Various levels of water route challenges were administered, depending on the experiment being conducted. Challenge doses ranged from less than one pfu/ml to 490,000 pfu/ml aquaria water. Controls received equal amounts of MEM-5% added directly to the aquaria water.

Confirmation of Viral Infection

All fish exposed to IHNV were observed for typical symptoms of the disease including anorexia, lethargy, exopthalmia, fecal casts, petechial hemorrhaging about fins and a generalized darkening of the dorsal areas of the body (Amend, 1974).

Dead fish from immunization experiments were not examined for the presence of viral agents because these fish were exposed to

either attenuated virus, wild type virus or both. All non-immunized control fish which died after being challenged were examined for the presence of virus. A final identification was made by observing the appearance of characteristic IHNV CPE.

EXPERIMENTAL RESULTS

Occurrence and Distribution of Fish Viruses

Anadromous Fishes

During this study, many stocks of anadromous salmonids were examined for the presence of viruses and for specific neutralizing antibodies against these agents. Figure 1 illustrates the geographic location of sites sampled from 1972-75.

Only one isolate of IPNV was detected from the examination of adult and juvenile anadromous salmonids during 1972-73 (Tables 3 and 4). Because McMichael (1973) had isolated and identified IPNV from returning adult coho salmon and their progeny at Bonneville Hatchery during 1971-72, adult fall chinook and coho salmon at this location were extensively examined. Repeated sampling at Bonneville during 1972-73 did not result in the isolation of any viral agents from adult or juvenile coho or fall chinook salmon. Blood samples from these fish, however, did contain anti-IPNV specific neutralizing antibodies.

Infectious pancreatic necrosis virus was isolated in 1972 from adult coho salmon returning to Cascade Hatchery. Six of the twelve pooled visceral samples examined contained the virus. Although offspring from this spawning were isolated and repeatedly sampled

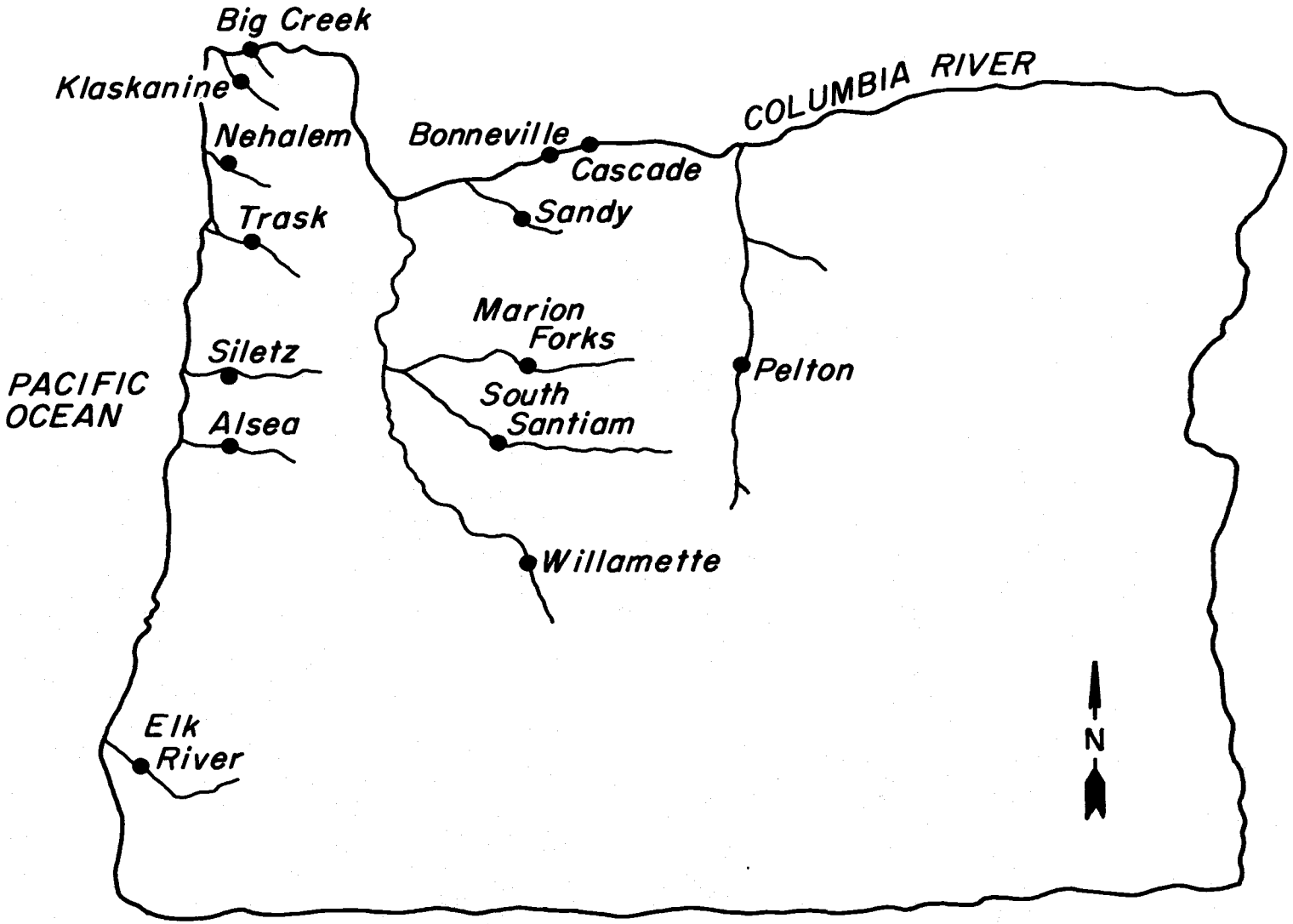

Figure 1. Location of selected sites where anadromous salmonids were sampled for viral examination.

Table 3. The 1972-73 virological examination of adult anadromous salmonids from selected locations in Oregon.

Hatchery location	Species sampled	Number of fish examined	Date of sampling	Virus recovered	Presence of specific neutralizing antibodies
Marion Forks	spring chinook	60	9-72	None	Not Tested
Marion Forks	spring chinook[a]	5	9-72	None	Anti-IPNV
Dexter	spring chinook	60	9-72	None	Not Tested
Bonneville	fall chinook	150	9-72	None	Anti-IPNV
Bonneville	coho	210	11-72	None	Anti-IPNV
Big Creek	fall chinook	60	9-72	None	Not Tested
Big Creek	coho	60	11-72	None	None
Big Creek	steelhead	60	1-73	None	None
Cascade	coho	60	10-72	IPNV	None
Sandy	coho	60	11-72	None	Anti-IPNV
Nehalem	coho	60	11-72	None	None
Klaskanine	coho	60	11072	None	None
Alsea	coho	60	11-72	None	None
Elk River	chinook	60	12-72	None	None

[a] Landlocked

Table 4. The 1972-73 virological examination of anadromous salmonid fry and juveniles from selected locations in Oregon.

Hatchery location	Species sampled	Number of fish examined	Date of sampling	Virus recovered	Presence of specific neutralizing antibodies
Marion Forks	spring chinook fry	50	2-73	None	Not Tested
Bonneville	fall chinook fry	50	12-72	None	Not Tested
Bonneville	coho fry	50	2-73	None	Not Tested
Big Creek	fall chinook fry	50	12-72	None	Not Tested
Big Creek	steelhead fry	50	4-73	None	Not Tested
Cascade	fall chinook fry	50	12-72	None	Not Tested
Cascade	coho fry	150	1-73 2-73	None	Not Tested
Cascade	coho juveniles	50	5-73	None	None
Sandy	coho fry	50	3-73	None	Not Tested
Nehalem	coho fry	50	3-73	None	Not Tested
Klaskanine	coho fry	50	3-73	None	Not Tested
Alsea	coho fry	50	3-73	None	Not Tested

for viral infection, no viral agents were recovered. Furthermore, blood samples collected from the adult and juvenile fish did not contain specific neutralizing antibodies against either IPNV or IHNV. No viral agents were recovered from any other populations of anadromous salmonids examined during 1972-73, but anti-IPNV antibodies were detected in adult coho salmon returning to Sandy Hatchery.

Two viral isolations occurred from the same stock of spring chinook returning to the Pelton Dam holding facility (Tables 5 and 6). Infectious hematopoietic necrosis virus was isolated in August, 1973 by members of the Bureau of Sports Fisheries and Wildlife at the Western Fish Disease Laboratory, Seattle, Washington. In September, 1973, IPNV was isolated from fish of the same returning population, though extensive examination of fry from this spawning group did not result in isolation of any viral agents. Other anadromous populations sampled during 1973-74 were negative for viral agents, and all blood samples examined for specific anti-viral antibodies during this period were also negative. No viral agents were isolated from any of the stocks examined during 1974-75 (Table 7).

During the summer of 1972, a number of anadromous fish were collected off the central Oregon coast. All samples examined for virus and all blood samples assayed for specific anti-viral antibodies were negative (Table 8).

Table 5. The 1973-74 virological examination of adult anadromous salmonids from selected locations in Oregon.

Hatchery location	Species sampled	Number of fish examined	Date of sampling	Virus recovered	Presence of specific neutralizing antibodies
Marion Forks	spring chinook	60	9-73	None	None
Bonneville	fall chinook	60	9-73	None	None
Bonneville	coho	60	10-73	None	None
Big Creek	fall chinook	60	10-73	None	None
Big Creek	coho	60	11-73	None	None
Cascade	fall chinook	60	9-73	None	None
Cascade	coho	60	10-73	None	None
Sandy	coho	60	10-73	None	None
Klaskanine	coho	60	11-73	None	None
Alsea	coho	60	11-73	None	Not Tested
Trask	coho	60	11-73	None	Not Tested
Siletz	coho	60	11-73	None	Not Tested
So. Santiam	steelhead	55	3-74	None	Not Tested
Pelton	spring chinook	30	9-73	IPNV	Not Tested
			8-73	IHNV[a]	Not Tested

[a] Infectious hematopoietic necrosis virus was isolated and identified earlier from stocks at this location by members of the Bureau of Sport Fisheries and Wildlife at the Western Fish Disease Laboratory, Seattle, Washington.

Table 6. The 1973-74 virological examination of anadromous salmonid fry and juveniles from selected locations in Oregon.

Hatchery location	Species sampled	Number of fish examined	Date of sampling	Virus recovered
Marion Forks	spring chinook fry	60	1-74	None
Bonneville	fall chinook juveniles	60	2-74	None
Bonneville	coho fry	60	2-74	None
Big Creek	fall chinook juveniles	60	1-74	None
Big Creek	coho fry	60	1-74	None
Cascade	fall chinook juveniles	60	2-74	None
Cascade	coho fry	60	2-74	None
Klaskanine	coho fry	60	1-74	None
Alsea	coho fry	60	3-74	None
Siletz	coho fry	60	3-74	None
Pelton	spring chinook fry	150	12-73 1-74	None

Table 7. The 1974-75 virological examination of adult anadromous salmonids from selected locations in Oregon.

Hatchery location	Species sampled	Number of fish examined	Date of sampling	Virus recovered
Bonneville	fall chinook	60	9-74	None
Bonneville	coho	60	11-74	None
Sandy	coho	60	11-74	None
Klaskanine	coho	60	11-74	None
Alsea	coho	60	11-74	None

Table 8. Summary of results obtained from the 1972 viral examination of anadromous salmonids off the Oregon coast. [a]

Species examined	Number of fish examined for viral agents	Virus recovered	Number of fish examined for specific neutralizing antibodies	Antibodies detected [b]
coho	103	None	23	None
chinook	15	None	2	None

[a] Samples were placed in five fish pools when possible.

[b] Blood was collected by means of cardiac puncture.

Examination of Salmonids from Central Oregon

In May and June of 1973, the Oregon Wildlife Commission's Fall River Hatchery experienced an epizootic in juvenile eastern brook trout (Salvelinus fontinalis) East Lake stock. A high mortality was associated with the symptoms of whirling, swimming in a horizontal spiraling manner, mucous deposits in the stomach and intestines, colorless spleen and liver and exophthalmia. The affected fish did not respond to antibiotic treatment and because no bacterial, protozoan or fungal pathogens were isolated, fish were examined for viral agents and in June, 1973, IPNV was isolated and identified from moribund animals. This isolation marked the first recorded viral epizootic in fish from Oregon. Subsequently, IPNV was isolated from all stocks of fish in the hatchery including two stocks of eastern brook trout (East Lake and Elk Lake-Big Lava Lake), as well as from yearling and juvenile rainbow trout (Table 9). Blood samples obtained from all groups contained specific anti-IPNV antibodies. However, only the East Lake stock of eastern brook trout suffered heavy losses. Fewer deaths occurred in the Elk Lake-Big Lava Lake stock of eastern brook trout and the juvenile and yearling rainbow trout were asymptomatic. Infectious pancreatic necrosis virus was isolated from eastern brook trout sampled downstream from the hatchery, but not from fish above the hatchery.

Table 9. The virological examination of Fall River hatchery stocks during 1973 Infectious Pancreatic Necrosis virus (IPNV) epizootic.

Species sampled	Number of fish examined	Date of sampling	Virus recovered	Presence of specific neutralizing antibodies
eastern brook trout juveniles East Lake stock	50	6-6-73	IPNV	Not Tested
eastern brook trout juveniles East Lake stock	20	6-12-73	IPNV	Not Tested
eastern brook trout juveniles Elk Lake-Lava Lake stock	20	6-12-73	IPNV	Not Tested
rainbow trout juveniles	20	6-12-73	IPNV	Not Tested
eastern brook trout juveniles East Lake stock	20	6-12-73	IPNV	Anti-IPNV
eastern brook trout juveniles Elk Lake-Lava Lake stock	20	6-15-73	IPNV	Anti-IPNV
rainbow trout juveniles	20	6-15-73	IPNV	Anti-IPNV
rainbow trout yearlings	60	6-15-73	IPNV	Anti-IPNV
eastern brook trout adults from Fall River downstream from the hatchery	4	6-15-73	IPNV	Not Tested
eastern brook trout juveniles from Fall River upstream from the hatchery	20	6-22-73	None	Not Tested
eastern brook trout juveniles from Indian Creek upstream from the hatchery	20	6-22-73	None	Not Tested

As a result of this epizootic, an examination of certain central Oregon salmonid populations for viral agents was undertaken in the summer of 1973. One purpose of this survey was to locate populations of IPNV-free eastern brook trout that might be used as future egg sources. Salmonids from other central Oregon locations were also sampled and examined for the presence of viral agents in order to determine the prevalence of fish viruses in this area (Figure 2). Infectious pancreatic necrosis virus was isolated and identified from eastern brook trout at Fish Lake, Big Lava Lake and Elk Lake (Table 10). Specific anti-IPNV antibody was detected in serum samples from these locations, as well as from eastern brook trout at Wizard Falls Hatchery.

On September 10, 1973, the Oregon Wildlife Commission treated Fall River and its tributaries with rotenone to poison the fish population inhabiting these waters. This procedure was carried out in conjunction with the disinfection of the hatchery facilities to eliminate all possible sources of IPNV from the hatchery and its surrounding watershed. Samples were obtained from dead fish in the river at several locations, and IPNV was isolated from eastern brook trout in the river downstream from the hatchery and in private dirt fishing ponds upstream from this fish rearing facility (Table 11). Viral agents were not isolated from rainbow trout or brown trout (S. trutta) inhabiting the river.

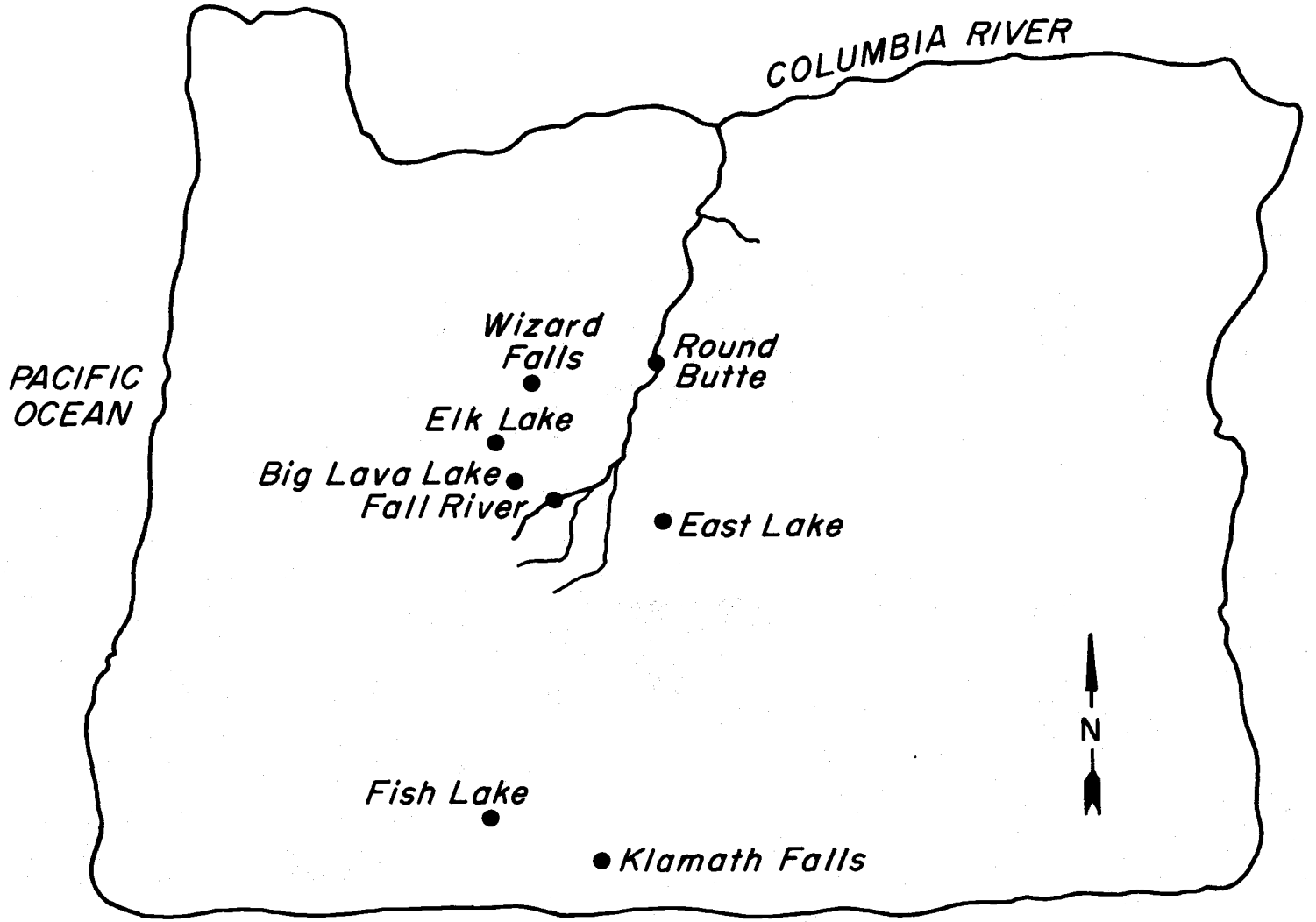

Figure 2. Location of selected sites in central Oregon where salmonids were sampled for viral examination.

Table 10. The virological examination of salmonids from selected central Oregon locations during 1973.

Sampling location	Species sampled	Number of fish examined	Date of sampling	Virus recovered	Presence of specific neutralizing antibodies
Big Lava Lake	eastern brook trout adults	7	6-29-73	None	None
Klamath Hatchery	brown trout juveniles, Suttle Lake stock	20	6-29-73	None	Not Tested
	brown trout juveniles, East Lake stock	20	6-29-73	None	Not Tested
East Lake	eastern brook trout adults	70	7-30-73	None	None
Wizard Falls Hatchery	eastern brook trout adults	24	7-30-73	None	Anti-IPNV
Round Butte Hatchery	chinook juveniles	25	8-15-73	None	Not Tested
Fish Lake	eastern brook trout adults	60	9-5-73	IPNV	Anti-IPNV
Big Lava Lake	eastern brook trout adults	60	9-12-73	IPNV	Anti-IPNV
Elk Lake	eastern brook trout adults	30	9-12-73	IPNV	Anti-IPNV

Table 11. The viral examination of salmonids collected at the time Fall River was treated with rotenone.

Sampling location	Species sampled	Number of fish examined	Date of sampling	Virus recovered
Fall River upstream from the hatchery	rainbow trout adults	5	9-10-73	None
	eastern brook trout adults	25	9-10-73	IPNV
Fall River at the hatchery	eastern brook trout adults	3	9-10-73	None
	rainbow trout adults	3	9-10-73	None
	brown trout adults	3	9-10-73	None
Fall River downstream from the hatchery	eastern brook trout adults	10	9-10-73	IPNV
	brown trout adults	5	9-10-73	None

Development of a Viral Vaccine Against Infectious Hematopoietic Necrosis Virus

Two strains of attenuated IHNV initiated by McMichael (1974) were tested further for immunizing ability in salmonid fishes. Because the attenuated IHNV water-borne vaccine appeared to be more promising as a simple, efficacious vaccine, this virus was tested more thoroughly than the attenuated IHNV injectable vaccine.

Determination of the Degree of Attenuation of Attenuated Infectious Hematopoietic Necrosis Virus Water-Borne Vaccine

The degree of attenuation of the attenuated IHNV water-borne vaccine strain was determined in sockeye salmon by comparing its pfu/LD_{50} ratio to the pfu/LD_{50} of the wild type IHNV. The attenuated IHNV water-borne vaccine was reduced in virulence over one-hundred times when compared to the wild type IHNV (Tables 12 and 13; Figure 3). The LD_{50} for wild type IHNV was calculated to be 1.26 pfu/fish, and from using data in Table 13, the LD_{50} of the attenuated IHNV water-borne vaccine strain was found to be 130 pfu/fish.

Determination of Water Route Challenge Levels Using Wild Type Infectious Hematopoietic Necrosis Virus

Water route challenges were determined by exposing fish to three levels of virulent wild type IHNV added directly to aquarium

Table 12. Base line kill curve data used for calculating the LD_{50} of the IHNV-71-Attenuated-water-borne viral vaccine strain when injected ip into sockeye salmon.[a]

Virus dilution	Number of deaths in each three day period after injection[b]							Number of deaths	Percent	Mean Day
	(1-3)	(4-6)	(7-9)	(10-12)	(13-15)	(16-18)	(19-21)	Number tested	deaths	to death
undiluted[c]	0	13	3	0	2	2	0	20/20	100	8
10^{-1}	0	9	3	2	0	1	0	15/20	75	8
10^{-2}	0	6	5	1	0	0	0	12/20	60	7
10^{-3}	0	2	6	4	0	0	0	12/20	60	9
10^{-4}	0	1	6	0	1	0	0	8/20	40	8
10^{-5}	0	0	2	1	2	2	0	7/20	35	12
10^{-6}	0	0	2	0	2	1	0	5/20	25	12
10^{-7}	0	0	0	0	0	0	0	0/20	0	
injected controls[d]	0	0	0	0	0	0	0	0/20	0	
non-injected controls	0	0	0	0	0	0	0	0/20	0	

[a] Mean weight of fish was 0.3 g.

[b] Each experimental fish received 0.015 ml of a viral dilution in HBSS.

[c] Titer of undiluted virus used for injection was 3.9×10^{7} pfu/ml.

[d] Control fish were injected with 0.015 ml of HBSS.

Table 13. Base line kill curve data used for calculating the LD_{50} of the wild type strain of IHNV when injected ip into sockeye salmon.[a]

Virus dilution[b]	Number of deaths in each three day period after injection[c] (1-3)	(4-6)	(7-9)	(10-12)	(13-15)	(16-18)	(19-21)	Number of deaths / Number tested	Percent deaths	Mean Day to death
10^{-3}	0	16	2	2	0	0	0	20/20	100	8
10^{-4}	0	9	7	2	1	0	0	19/20	95	7
10^{-5}	0	5	7	1	2	1	0	16/20	80	9
10^{-6}	0	2	1	0	3	0	2	8/20	40	12
10^{-7}	0	0	0	0	0	0	0	0/20	0	
Injected controls[d]	0	0	0	0	0	0	0	0/20	0	
Non-injected controls	0	0	0	0	0	0	0	0/20	0	

[a] Mean weight of fish was 0.3 g.

[b] Each experimental animal received 0.015 ml of a viral dilution in HBSS.

[c] Titer of the undiluted virus used for injection was 5.4×10^7 pfu/ml.

[d] Control fish were injected with 0.015 ml of HBSS.

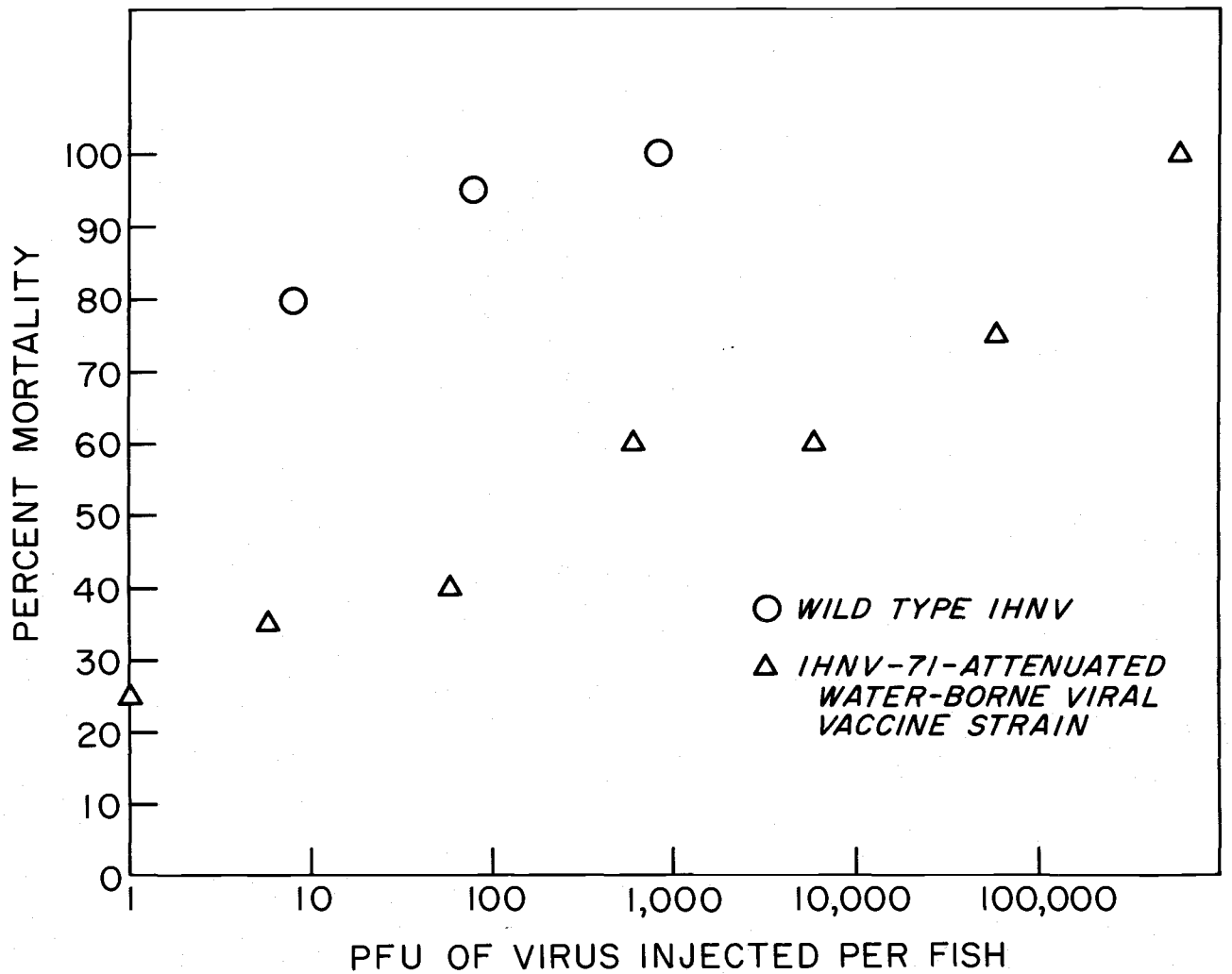

Figure 3. In vivo comparison of the virulence of IHNV-71-Attenuated-water-borne viral vaccine strain to wild type IHNV.

water for an exposure period of 48 hours. Each of the three levels of wild type IHNV administered as a water route challenge killed over 90% of the fish in the experimental groups (Table 14).

Determination of Efficacy of Immunization with Attenuated Infectious Hematopoietic Necrosis Virus Water-Borne Vaccine

The attenuated IHNV water-borne vaccine was tested solely as a water-borne immunizing agent in a series of experiments designed to develop a simple, efficient method of vaccine administration. This vaccine elicited a strong protective response in fish immunized with it (Table 15). All three immunization doses protected at least 97% of the vaccinated fish which received an ip challenge. Only three percent of the non-immunized control fish survived the same challenge.

This attenuated water-borne viral vaccine, administered at selected levels, also protected immunized salmon to two concentrations of water route challenge, as well as challenge by ip injection of virus (Table 16). Each group of vaccinated animals effectively withstood these three challenges. The two lower immunizing doses of 6,200 and 12,400 pfu/ml aquarium water protected over 90% of the fish challenged and 87% of fish immunized with the highest dose of 18,600 pfu/ml aquarium water survived. However, 12% of the fish immunized with the highest dose died from viral infection during

Table 14. Determination of water route challenge levels in kokanee salmon[a] with wild type IHNV.

Plaque forming units added per ml aquarium water (pfu/ml)[b]	Number of deaths / Number tested	Percent deaths	Mean Day to death
1, 200	18/20	90	10. 3
2, 400	19/20	95	10. 5
4, 800	19/20	95	11. 1

[a]Mean weight of fish was 0. 77 g.

[b]Exposure levels consisted of undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours.

Table 15. Efficacy of immunization of sockeye salmon fry[a] with IHNV-71-Attenuated-water-borne viral vaccine after an injected ip challenge[b] with 48 x LD_{50} of virulent wild type IHNV.

Immunizing dose[c] (pfu/ml aquarium water)	Number of survivors / Number tested	Percent survivors	Mean Day to death
1,500	39/40	97.5	10
7,500	39/40	97.5	12
15,000	40/40	100.0	--
Controls[d, e]	1/40	2.5	8

[a]Mean weight of fish was 0.4 g.

[b]Challenge contained 60 pfu in 0.02 ml of HBSS injected ip 25 days after immunization.

[c]Immunization dose consisted of undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours.

[d]Non-immunized control fish were challenged with 48 x LD_{50} of wild type IHNV injected ip.

[e]Another control group received an immunization dose of 15,000 pfu/ml aquarium water but was not challenged - all fish survived exposure to this immunization dose.

Table 16. Efficacy of three different immunization doses of IHNV-71-Attenuated-water-borne viral vaccine to three levels of challenge in sockeye salmon juveniles.[a]

Immunizing dose[b] (pfu/ml aquarium water)	Number of survivors/Number tested following challenge[c] with		
	46 x LD_{50} injected ip	2,000 pfu/ml aquarium water	4,000 pfu/ml aquarium water
6,200	19/20 (95%)	20/20 (100%)	19/20 (95%)
12,400	20/20 (100%)	16/20 (80%)	20/20 (100%)
18,600	13/15 (87%)	16/18 (89%)	14/20 (70%)
Non-immunized controls[d]	0/20 (0%)	3/20 (15%)	2/20 (10%)

[a] Mean weight of fish was 0.9 g.

[b] Immunization doses consisted of undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours.

[c] Injected challenges were made in 0.02 ml HBSS and water borne challenges contained undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours. Both were administered 21 days after immunization.

[d] Another control group received an immunization dose of 18,600 pfu/ml aquarium water, but was not challenged - eighty percent of the fish survived exposure to this immunization dose.

immunization.

Determination of Minimum Immunizing Levels in Sockeye Salmon

The minimum immunizing dose of the attenuated IHNV water-borne vaccine was determined by administering five vaccine concentrations to fish and challenging them by ip injection with four concentrations of wild type IHNV (Table 17). An immunizing dose of 7,500 pfu/ml aquarium water was the minimum level that provided protection to at least 90% of fish challenged with 460 x LD_{50} and 65% of the fish challenged with up to 46,000 x LD_{50} of wild type IHNV injected ip.

Comparison of Different Exposure Periods to Attenuated Infectious Hematopoietic Necrosis Virus Water-Borne Vaccine

Six different exposure periods were tested using three immunizing doses in sockeye salmon to determine the best combination of the two for use in immunization studies. Results listed in Table 18 clearly indicate that a 48 hour exposure period to the immunizing agent provided the best overall protection with the three immunizing doses. However, exposure periods of 12 hours and 24 hours resulted in good protection, while the shorter periods were not effective. An exception to this occurred with an exposure period of 15 minutes to an immunizing dose of 49,000 pfu/ml aquarium water which resulted in 69% of the fish surviving the challenge.

Table 17. Efficacy of five different immunizing doses of IHNV-71-Attenuated-water-borne viral vaccine to four injected ip challenge levels of wild type IHNV when administered to juvenile sockeye salmon.[a]

Immunizing doses[b] (pfu/ml aquarium water)	Number of survivors/Number tested following injected ip challenge[c] with							
	46 x LD_{50}	MDD[d]	460 x LD_{50}	MDD	4,600 x LD_{50}	MDD	46,000 x LD_{50}	MDD
7500	15/20 (75%)	10.80	18/20 (90%)	9.00	14/20 (70%)	10.20	13/20 (65%)	14.40
750	9/20 (45%)	9.50	14/20 (70%)	9.50	7/20 (35%)	8.50	9/20 (45%)	9.50
75	4/20 (20%)	6.25	3/20 (15%)	6.82	2/20 (10%)	6.88	0/20 (0%)	7.73
7.5	1/20 (5%)	6.00	2/20 (10%)	7.27	3/20 (15%)	5.11	0/20 (0%)	5.40
1	1/20 (5%)	6.84	0/20 (0%)	5.95	0/15 (0%)	6.13	0/19 (0%)	4.36
Non-immunized controls[e]	0/20 (0%)	7.70	0/20 (0%)	5.05	0/20 (0%)	4.85	0/21 (0%)	3.35

[a] Mean weight of fish was 0.95 g.

[b] Immunization dose consisted of tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours.

[c] Challenge doses were injected in 0.02 ml of HBSS 25 days after immunization.

[d] Mean Day to death.

[e] Another control group received an immunization dose of 7,500 pfu/ml aquarium water, but was not challenged - all fish survived exposure to this immunization dose.

Table 18. Efficacy of immunization of IHNV-71-Attenuated-water-borne viral vaccine using six different exposure periods with three immunizing levels to water route challenge[a] with wild type IHNV in kokanee salmon juveniles.[b]

Immunizing doses[c] (pfu/ml aquarium water)	Number of survivors/Number tested after receiving immunization doses for the following exposure periods					
	0.25 hours	0.5 hours	1 hour	12 hours	24 hours	48 hours
3,500	2/20 (10%)	6/20 (30%)	11/18 (61%)	15/19 (79%)	13/20 (65%)	18/20 (90%)
10,500	1/20 (5%)	5/20 (25%)	9/20 (45%)	14/17 (82%)	19/20 (95%)	16/18 (89%)
49,000	13/19 (69%)	11/20 (55%)	7/20 (35%)	16/18 (89%)	13/18 (72%)	17/19 (89%)
non-immunized control[d]	--	-	-	-	-	1/20 (5%)

[a] Challenge levels consisted of undiluted tissue culture fluid containing 2,400 pfu/ml added to ten liters of aquarium water for an exposure period of 48 hours.

[b] Mean weight of fish was 0.77 g.

[c] Immunization doses consisted of undiluted tissue culture fluid added to ten liters of aquarium water for the appropriate exposure period.

[d] Another control group received an immunization dose of 49,000 pfu/ml aquarium water for an exposure period of 48 hours, but was not challenged - all fish survived exposure to that immunization dose.

Efficacy of Immunization of Attenuated Infectious Hematopoietic Necrosis Virus Water-Borne Vaccine to Challenges with Four Different Strains of Virulent Virus

The attenuated IHNV water-borne vaccine provided protection against challenges with four different virulent IHNV strains isolated from locations in the western United States (Table 19). In this experiment, fish received two immunizing doses. The first contained IHNV at a concentration of 900 pfu/ml; and the second contained 240,000 pfu/ml aquarium water and was administered 32 days later. Although the challenge viruses were demonstrated to be virulent, the immunization dose protected at least 85% of the fish vaccinated. Only the OSV challenge virus failed to kill 75% or more of the non-immunized control fish.

Efficacy of Immunization with Attenuated Infectious Hematopoietic Necrosis Virus Water-Borne Vaccine 110 Days After Immunization

The protection afforded kokanee salmon fry by vaccination with the attenuated IHNV water-borne vaccine was shown to have a duration of at least 110 days (Table 20). Eighty-six percent of fish immunized with 14,000 and 21,000 pfu/ml, and 72% of those immunized with 7,000 pfu/ml survived the water route challenge administered 110 days after immunization. The non-immunized controls were susceptible to the challenge virus after being held 110 days.

Table 19. Efficacy of immunization of sockeye salmon[a] with IHNV-71-Attenuated-water-borne viral vaccine to water route challenges with four virulent isolates of IHNV from the western United States.

Immunizing doses[b] (pfu/ml aquarium water) Day 1-900 Day 32-240,000	Number of survivors/Number tested following challenges[c] with							
	SRCV 66,000 pfu/ml	MDD[d]	Wild Type IHNV 4,000 pfu/ml	MDD	Washington Cedar River 10,000 pfu/ml	MDD	OSV 36,300 pfu/ml	MDD
Experimental fish	37/40 (92.5%)	16.00	37/40 (92.5%)	17.00	37/40 (85%)	15.80	39/40 (97.5%)	11.00
Non-immunized controls[e]	4/20 (20%)	9.56	5/20 (25%)	10.90	1/20 (5%)	9.42	8/20 (40%)	9.00

[a] Mean weight of fish was 1.3 g.

[b] Each immunizing dose consisted of undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours.

[c] Challenge levels consisted of undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours 46 days after immunization.

[d] Mean Day to death.

[e] Another control group was immunized in the same manner as the experimentals but was not challenged - all fish survived exposure to this immunization dose.

Table 20. Efficacy of immunization of kokanee salmon fry[a] with IHNV-71-Attenuated-water-borne vaccine to a water route challenge[b] containing wild type IHNV 110 days after immunization.

Immunizing doses[c] (pru/ml aquarium water)	Number of survivors / Number tested	Percent survivors
7,000	26/36	72
14,000	30/35	86
21,000	31/36	86
Non-immunized controls[d]	1/18	6

[a] Mean weight of fish used was 0.77 g.

[b] Challenge level consisted of undiluted tissue culture fluid added to ten liters of aquarium water at a concentration of 2,400 pfu/ml for an exposure period of 48 hours, 110 days after immunization.

[c] Immunizing dose consisted of undiluted tissue culture fluid added to ten liters of aquarium water for an exposure period of 48 hours.

[d] Another control group received an immunization dose of 21,000 pfu/ml aquarium water but was not challenged - all fish survived exposure to this immunization dose.

Efficacy of Immunization with Attenuated Infectious Hematopoietic Necrosis Virus Water-Borne Vaccine in Chinook Salmon

When vaccinated with attenuated IHNV water-borne vaccine, chinook salmon were not afforded as much protection as kokanee or sockeye salmon (Table 21). The best combination of immunizing dose and exposure period resulted in 72% of the fish surviving the injected ip challenge. The 48 hour exposure period was again most effective when combined with any of the three immunizing doses.

Vacuum Infiltration Method of Immunization with Attenuated Infectious Hematopoietic Necrosis Virus Injectable Vaccine

While the vacuum infiltration method of vaccination with the attenuated IHNV injectable vaccine provided some protection, it was not nearly as effective as the attenuated IHNV water-borne vaccine. The immunization of kokanee and chinook salmon resulted in similar levels of protection by using this method (Tables 22 and 23). Sixty-three percent of the kokanee vaccinated with the highest immunizing dose of 770,000 pfu/ml were protected to the challenge level, whereas, the lower immunizing doses protected 50% or less of the experimental fish. An immunizing dose of 385,000 pfu/ml of attenuated virus provided the best protection to chinook juveniles immunized by this method, protecting 53% of the animals to the injected challenge.

Table 21. Efficacy of immunization of chinook salmon[a] using different exposure periods with three various immunization doses of IHNV-71-Attenuated-water-borne viral vaccine to injected ip challenge[b] containing SRCV.

Immunizing doses[c] (pfu/ml aquarium water)	Number of survivors/Number tested after receiving immunization doses for the following exposure periods			
	6 hours	12 hours	24 hours	48 hours
35,000	5/20 (25%)	6/19 (32%)	10/20 (50%)	11/19 (58%)
100,500	6/20 (30%)	14/20 (70%)	9/19 (47%)	13/18 (72%)
490,000	7/20 (35%)	13/19 (68%)	12/18 (67%)	12/19 (63%)
Non-immunized controls[d]	--	--	--	6/19 (32%)

[a]Mean weight of fish was 0.67 g.

[b]Challenges consisted of 180 pfu in 0.02 ml of EBSS injected ip 25 days after immunization.

[c]Immunizing doses consisted of undiluted tissue culture fluid added to ten liters of aquarium water for the appropriate exposure periods.

[d]Another control group received an immunization dose of 490,000 pfu/ml aquarium water but was not challenged - eighty percent of these fish survived exposure to that immunizing dose.

Table 22. The vacuum infiltration method of immunization of kokanee salmon[a] with IHNV-71-Attenuated-injectable viral vaccine.

Immunizing doses infiltrated[b] (pfu/ml in desiccator)	Number of survivors/Number tested following water route challenge[c] with wild type IHNV	Percent survivors	Mean Day to death
7,000	6/20	30	9.80
77,000	7/19	37	9.65
385,000	10/20	50	12.10
770,000	12/19	63	13.20
Non-immunized controls[d]	2/20	10	10.00

[a]Mean weight of fish was 0.68 g.

[b]Immunizing doses consisted of undiluted tissue culture fluid added directly to one liter of dechlorinated water in the vacuum desiccator.

[c]Challenge level consisted of undiluted wild type IHNV added directly to aquarium water at a concentration of 2,400 pfu/ml for an exposure period of 48 hours, 46 days after immunization.

[d]Another control group received an immunizing dose of 770,000 pfu/ml of vaccine infiltrated but was not challenged - all fish survived this immunization procedure.

Table 23. The vacuum infiltration method of immunization of chinook salmon[a] with IHNV-71-Attenuated-injectable viral vaccine.

Immunizing doses infiltrated[b] (pfu/ml in desiccator)	Number of survivors/Number tested following injected ip challenge[c] with SRCV	Percent survivors	Mean Day to death
7,000	7/20	35	10.2
77,000	4/20	20	10.8
385,000	8/15	53	12.4
770,000	9/19	47	14.4
Non-immunized controls[d]	4/20	20	12.4

[a]Mean weight of fish was 0.67 g.

[b]Immunizing doses consisted of undiluted tissue culture fluid added directly to one liter of dechlorinated water in the vacuum desiccator.

[c]Challenge level consisted of 180 pfu of SRCV injected ip 46 days after immunization.

[d]Another control group received an immunizing dose of 770,000 pfu/ml of vaccine infiltrated but was not challenged - all fish survived this immunization procedure.

DISCUSSION

After the isolation and identification of IHNV and IPNV from Oregon salmonids in 1971 (McMichael, 1974), extensive studies were undertaken to determine the occurrence, distribution and significance of these viruses in Oregon. Maintaining healthy stocks of salmonid fishes is of both commercial and recreational importance in Oregon and a knowledge of the occurrence and distribution of these viruses, as well as prevention and control of these viral diseases is helpful in achieving that goal. At the outset of this research, no methods were available for preventing IHNV outbreaks and the only reported method for controlling IHNV involved elevating water temperatures (Amend, 1970). Therefore, steps were taken to evaluate the vaccine first tested in this laboratory for immunization against IHNV.

The three viruses isolated from anadromous fish during this study were from animals which showed no evidence of viral pathology and were considered asymptomatic carriers. The isolation and identification of IPNV from fish at the Bonneville Hatchery in 1971 and Cascade Hatchery in 1972, along with the detection of anti-IPNV antibody in certain stocks, indicated that the Columbia River coho salmon might be asymptomatically carrying IPNV at a high incidence level. However, repeated examination of those fish for the next three years failed to detect any viral agents. Within the period of one

month, both IPNV and IHNV were isolated from the same population of returning adult spring chinook at Pelton Dam on the Deschutes River in central Oregon. Again, the viruses were not detected in the progeny and no mortality was associated with these isolations. Anti-IPNV antibodies were present in landlocked spring chinook from Detroit Reservoir on the North Santiam River in the same watershed from which McMichael isolated IPNV from wild cutthroat trout.

It is difficult to determine the significance of these isolations of salmonid viruses from anadromous populations. No mortality has been associated with any of them and the incidence was low. It is apparent that the Columbia River and its tributaries contain salmonid populations which are asymptomatically carrying these viral agents. It is interesting to note that no viruses have been isolated from any salmonids inhabiting or returning to Oregon coastal streams. A policy recently developed prohibiting the transfer of fish from the Columbia River and its tributaries to the coastal streams will help keep viral agents from watersheds where they have not been detected.

The isolation and identification of IPNV from juvenile eastern brook trout at Fall River Hatchery was the first documented epizootic in Oregon. The virus was also isolated and identified from fish inhabiting Fall River below the hatchery indicating that virus containing effluent from ponds containing infected fish was transmitting it to wild fish downstream. Since IPNV was isolated from all stocks of

fish at the hatchery, the Oregon Wildlife Commission decided to destroy all stocks of fish at this location and to treat the river to poison all fish inhabiting these waters. Three months after the epizootic and at the time the river was poisoned, IPNV was isolated from adult brook trout both upstream and downstream from the hatchery, demonstrating that infected fish were still present in the river. At the hatchery, 1. 2 million eastern brook trout juveniles, 576, 600 rainbow trout fingerlings and 71, 400 legal size rainbow trout were destroyed. After the river was poisoned and the hatchery was disinfected, rainbow trout were reared in the pond that previously contained East Lake stock of eastern brook trout. Repeated examination of these fish for viral agents and anti-viral antibodies were negative. Eastern brook trout fry hatched in the hatchery house following disinfection also were sampled on numerous occasions with no viral agents detected. These results indicate that the disinfection of the hatchery and poisoning of fish in the river aided in the elimination of the virus.

Following the epizootic at Fall River hatchery, a number of central Oregon salmonid populations were sampled and examined for viral agents to locate an IPNV-free eastern brook trout egg source and to determine the occurrence and distribution of salmonid viruses in central Oregon. Infectious pancreatic necrosis virus was found to be widespread, although eastern brook trout examined at East Lake,

the source of the eggs of the fish which suffered heavy losses during the epizootic, resulted in no viral isolations.

It has been demonstrated that central Oregon populations of eastern brook trout are infected with IPNV at a high incidence and for the first time, this virus has resulted in an epizootic in a hatchery facility. The source of these infected fish responsible for the Fall River Hatchery epizootic has not been clearly determined. It is significant that no viral agents have been found in eastern brook trout from East Lake. Since this stock of fish suffered the heaviest losses during the epizootic, they were implicated as the source of the virus. However, isolations of IPNV from eastern brook trout in both Elk and Big Lava Lakes have complicated the situation since their stocks of fish in the hatchery did not suffer nearly as heavy a mortality as the East Lake stock. The source of IPNV in central Oregon will probably never be determined because of the high incidence in eastern brook trout. Furthermore, the constant moving and stocking of fish from one location to another makes it difficult to identify the source of the virus. Infectious pancreatic necrosis virus in eastern brook trout populations of central Oregon has posed a serious threat to culture of this species.

Since IHNV and IPNV have been isolated from epizootics in several states, an interest has been taken in the development of viral vaccines against these viruses. McMichael's work (1974) initiated the

development of two attenuated strains of IHNV, one that could be used as a water-borne vaccine and the other to be administered by ip injection. Development of a live modified water-born viral vaccine was pursued for the following reasons: (1) a water-borne vaccine is easily administered; (2) attenuated viral vaccines that are used in human and veterinary medicine produce effective, long lasting immunity; (3) presumably, an attenuated viral water-borne vaccine produces local immunity which better protects the fish to infection encountered naturally; (4) a live attenuated water-borne vaccine would require fewer virus particles than a killed injected preparation and could thus be produced more economically.

The attenuated IHNV water-borne vaccine was extensively tested during these studies. The information gathered from these experiments supports the four statements listed above. Because the attenuated IHNV water-borne vaccine strain was shown to be one-hundred three times less virulent than the wild type IHNV, it is sufficiently modified to allow its use as a water-borne vaccine preparation. The method by which this vaccine confers immunity has not been determined. It is likely that upon addition of the attenuated virus to the water source, the fish encounters the virus and subsequently undergoes a mild infection which results in the production of immunity.

During these studies, a water route challenge was developed in an effort to more closely simulate a natural viral infection. It is

felt that this method of artificial challenge is better than exposing animals by ip injection of the agent.

Using both these types of challenges, vaccination with attenuated IHNV water-borne vaccine was found to be protective. Levels of immunity developed after vaccination varied with the immunization dose and size of fish vaccinated. The immunizing ability of attenuated IHNV water-borne vaccine was best exemplified in the experiment in which an immunizing dose containing 7,500 pfu/ml aquarium water for an exposure period of 48 hours protected 65% of the vaccinated fish to an injected ip challenge of 46,000 x LD_{50}.

Exposure periods to the immunizing virus during the vaccination procedure were important factors in levels of immunity produced. The 48 hour exposure period was the most effective in experiments using kokanee and chinook salmon. Exposure periods of 12 and 24 hours were also effective, but required a higher immunizing dose than the 48 hour exposure period to achieve the same levels of immunity. A short exposure period of 15 minutes is ineffective except at a very high immunizing dose.

The immunity developed by sockeye salmon after vaccination with attenuated IHNV water-borne vaccine was protective to water route challenges with several isolates from the western United States. Since only one antigenic type of IHNV is known, the immunity conferred by this preparation should offer protection against IHNV strains

isolated from a wide geographical range.

Most viral epizootics occur in fry or juvenile fish. The fish seem to be less resistant to viral infection during this period though, as they get larger, their susceptibility decreases. Thus, it is beneficial to be able to immunize fish at a very young age and protect them during the susceptible period. It is, therefore, necessary to have a vaccine that confers immunity for an extended period of time. Sockeye salmon juveniles vaccinated with the attenuated IHNV water-borne vaccine were resistant to a water route challenge 110 days after immunization. It was not determined whether the immunity lasted longer than 110 days but this duration of immunity does indicate the vaccine confers immunity throughout the animal's most susceptible age.

The immunization of chinook salmon with the water-borne vaccine was not as successful as the vaccination experiments in kokanee and sockeye, even though the chinook received much higher immunization doses. In preliminary experiments not reported in this study, chinook salmon were found to be more resistant than sockeye salmon to infection with wild type IHNV when exposed by the water route. It is therefore likely that chinook would also be more resistant to the immunizing effects of the attenuated IHNV water-borne vaccine.

During the course of one in vivo immunization study, a high level immunizing dose of attenuated IHNV water-borne vaccine caused

symptoms of viral infection and a 12% mortality of the vaccinated sockeye salmon. This indicates that the modified virus was virulent enough to cause death in some animals. However, in subsequent experiments using much higher immunizing doses, this phenomenon was not observed. For some reason, the juvenile sockeye salmon that died as a result of vaccination were not capable of surviving infection with the attenuated virus.

The vacuum infiltration method of vaccination was effective when the attenuated IHNV injectable vaccine was used as the immunizing agent. Fish immunized in this manner did not suffer any adverse effects and 63% of the kokanee and 53% of the chinook vaccinated were protected. The method by which the vaccine is delivered to the fish is not known. The return to atmospheric pressure after the vacuum is released presumably forces the uptake of the vaccine.

Some important aspects of the attenuated IHNV water-borne vaccine and the immune response it produces have not yet been determined. Three of these areas that deserve future consideration in experimental work are: (1) the mechanism of the immune response, (2) determination of whether the attenuated virus will regain virulence after several passages in susceptible hosts, (3) determination of viral replication in animals immunized with the attenuated vaccine.

It should be noted that all the in vivo studies were performed under conditions unlike large scale hatchery production situations in

which a vaccine preparation would be used. The further development of the water-borne vaccine must include large scale testing and adjustments in the vaccination procedure may be required to duplicate the results obtained under controlled experimental conditions. Some parameters that may influence the immunizing ability of the vaccine preparation are environmental factors such as fluctuation in water temperature and the presence of other disease agents. However, the ease of administration of this vaccine preparation, as compared to injection or other vaccination methods, makes it a desirable immunizing agent for fish reared in large scale production facilities.

The attenuated IHNV water-borne vaccine has been demonstrated to be efficacious, potent and easily administered. Even though the experiments described in this work are only preliminary, the attenuated IHNV water-borne vaccine may offer a method for the future prevention and control of IHNV in salmonid fishes.

SUMMARY AND CONCLUSIONS

1. Two isolates of IPNV and one IHNV strain were recovered and identified from samples collected from anadromous Oregon salmonids during the period 1972-75. All isolations occurred from adult fish returning to the Columbia River and its tributaries.
2. All viruses from anadromous fish were recovered in apparently healthy, asymptomatic carrier fish and no mortality was associated with any of these fish.
3. Viral isolations, as well as the detection of anti-IPNV antibody from fish in the Santiam and Columbia Rivers systems, indicate the possible existence of viral carrier states in populations from these watersheds.
4. No viral agents were detected from any fish returning to Oregon coastal streams.
5. Infectious pancreatic necrosis virus was isolated and identified from an epizootic in eastern brook trout at Fall River Hatchery in central Oregon. This is the first documented viral epizootic in fish from Oregon caused by IPNV.
6. Infectious pancreatic necrosis virus was isolated from eastern brook trout at several central Oregon locations indicating that this agent is present at a high incidence. Infectious pancreatic

necrosis virus poses a grave threat to the culture of eastern brook trout in Oregon.

7. A water route challenge with wild type IHNV was developed to more closely simulate a natural viral infection.
8. The attenuated IHNV water-borne vaccine virus was shown to be 103 times less virulent than the wild type strain from which it was derived.
9. The attenuated IHNV water-borne vaccine was demonstrated to be an effective water-borne vaccine against IHNV disease in salmonid fish.
10. A 48 hour exposure period to the attenuated IHNV water-borne vaccine was demonstrated to be most effective in conferring immunity.
11. Vaccination with the attenuated IHNV water-borne vaccine protected immunized fish to high levels of IHNV administered by a water route or injected ip challenge.
12. Vaccination with the attenuated IHNV water-borne vaccine was protective to challenges with several virulent IHNV strains isolated from salmonids in the western United States.
13. The immunity developed to this attenuated virus was protective to challenge 110 days after vaccination.
14. Kokanee and sockeye salmon are more effectively immunized than chinook salmon.

15. The vacuum infiltration method of immunization using the attenuated IHNV injectable vaccine effectively vaccinated kokanee and chinook salmon. However, this method of immunization was not as effective as the attenuated IHNV water-borne vaccine.

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