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Title: COMPARATIVE SUSCEPTIBILITY OF SALMONID FISH AND SALMONID FISH CELL LINES TO FIVE ISOLATES OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS, AND BIOLOGICAL PROPERTIES OF TWO PLAQUE VARIANTS

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The virulence of low-passage isolates of infectious hematopoietic necrosis virus (IHNV) was compared in juvenile chinook (Oncorhynchus tshawytscha), sockeye (kokanee) (Oncorhynchus nerka) and coho salmon (Oncorhynchus kisutch), and rainbow trout (steelhead) (Salmo gairdneri). The 50% lethal dose was determined after waterborne exposure of fish to different concentrations of virus and used as an index of virulence. All isolates tested were pathogenic in sockeye salmon and rainbow trout, but two isolates were comparatively avirulent in chinook salmon. Infection of coho salmon with IHNV was demonstrated, however this species appeared to be

more resistant than other salmonid species tested. Hybrids of IHNV-resistant coho salmon with susceptible rainbow trout were shown to be resistant.

The replication of different IHNV isolates was compared in cell lines derived from the fish species tested *in vivo*. Efficiency of plaquing, multicyclic growth curves and endpoint dilution assays showed that the different susceptibility of host species to IHNV isolates could be demonstrated at the cellular level.

Large-plaque and small-plaque variants of IHNV were partially purified by cloning. The small-plaque variant was numerically dominant in low-passage virus stocks, unpassaged virus from natural sources, and virus recovered from experimentally-infected fish, thus suggesting that it is the virulent, wild-type form. The difference in the plaque size of the two variants appeared to result from inhibition of the small plaque variant by acidity of the overlay.

Comparative susceptibility of salmonid fish and salmonid fish cell lines to five isolates of infectious hematopoietic necrosis virus, and biological properties of two plaque variants.

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INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a highly destructive pathogen of juvenile salmonid fishes. Evidence from both naturally occurring outbreaks of the disease and experimental exposure of fish to virus indicates that isolates from sockeye salmon (Oncorhynchus nerka) and rainbow trout (Salmo gairdneri) possess reduced virulence for salmonid species other than their natural host (Pilcher and Fryer, 1980). However, an isolate from dying chinook salmon (Oncorhynchus tshawytscha) was found to be virulent in sockeye salmon and in both anadromous rainbow trout (steelhead) and nonmigratory rainbow trout (Wingfield and Chan, 1970). Coho salmon (Oncorhynchus kisutch) are considered resistant to IHNV (Wingfield et al., 1970; Wingfield and Chan, 1970).

This study was generated in part by the need for information on host susceptibility to IHNV isolates. The increasing incidence of the disease in the Pacific Northwest has accentuated the need to determine possible effects of transfer of fish infected with virus to virus-free areas, or to areas in which virus is known to occur (Groberg et al., 1982).

The relative susceptibility of different stocks of a salmonid species to IHNV has not been thoroughly investigated, although variation in the susceptibility of chinook salmon stocks has been reported (Wertheimer and Winton, 1982). Resistance to IHNV is genetically influenced (McIntyre and Amend, 1978), but the nature of the factor(s) controlling resistance has not been defined. However, a sockeye salmon cell line (SSE-5) was more susceptible than a chinook salmon cell line (CHSE-114) to infection by a virus isolated from sockeye salmon, suggesting that resistance may be determined at the cellular level (Nims et al., 1969).

The study of plaque mutants of animal viruses has shown that although small plaque variants result from a variety of causes, they are usually attenuated (Takemoto, 1966). An attenuated IHNV preparation can protect fish from a virulent virus challenge (Fryer et al., 1976); however, such a preparation has the potential for reversion or back mutation to virulence. An understanding of viral genetics through the study of plaque mutants has made possible measurement of reversion in vitro (Vogt et al., 1957). Knowledge of the plaque size variation in IHNV might be used to improve the homogeneity and determine the genetic stability of attenuated virus preparations, and to distinguish between wild and attenuated virus.

The purpose of this study was to: (1) determine the host specificity of IHNV by measuring the virulence of low passage isolates for different salmonid species, (2) develope an *in vitro* model of host specificity based on the infectivity of low passage isolates for cell lines derived from different salmonid species, (3) hybridize resistant salmonid fish species with susceptible species to determine if resistant hybrids can be produced, (4) examine low passage and unpassed virus by plaque assay to define an *in vivo* role of plaque size, (5) purify by plaque cloning IHNV populations producing large and small plaques and (6) provide further information on *in vitro* parameters affecting plaque size.

LITERATURE REVIEW

External and Internal Signs

Acute epizootics caused by IHNV are characterized by rapid and often high mortality. Affected fish may show a variety of signs including dark color, exopthalmia, abdominal distension, fecal casts, hemorrhages at the base of the fins and between spaces of the lateral musculature, and behaviour alternating between lethargy and frenzy (Rucker et al., 1953; Ross et al., 1960; Amend et al., 1969).

Internal signs of the disease include a fluid-filled digestive tract, hemorrhage of the mesenteries and a pale color of the liver, spleen and kidney indicating anemia (Rucker et al., 1953, Ross et al., 1960). Microscopically, severe necrosis of the hematopoietic tissues of the anterior kidney and spleen is characteristic of the disease. Necrosis of the liver and the lining of the gastrointestinal tract can also be observed (Yasutake et al., 1965; Amend et al., 1969).

Chronic epizootics with low mortality may occur as fish increase in size. Infected fish may exhibit lordosis, scoliosis, head deformities, gastrointestinal signs and areas of fungal growth at the base of fins, indicating necrosis of external tissue (Rucker et al., 1953).

Distribution and Host Susceptibility

The geographic range where IHNV is endemic includes the Pacific coast of North America. Transfer of infected fish and fish eggs may have been responsible for recent epizootics in Minnesota, Montana, Idaho and South Dakota (Pilcher and Fryer, 1980). The virus has become widely distributed in Japan following introduction of fish eggs from Alaska (Sano et al., 1977).

A disease of juvenile sockeye salmon (Oncorhyncus nerka) in Washington State which was not influenced by antibiotics and was transmissable by filterable agent was reported by Rucker et al. (1953). The agent was not isolated in cell culture but probably was a strain of IHNV (Pilcher and Fryer, 1980). The species-specific nature of the agent was demonstrated. Juvenile chinook salmon (Oncorhynchus tshawytscha) and sockeye salmon of similar size were placed in water downstream of infected fish. The sockeye salmon suffered a 95% mortality while only 5% of the chinook salmon died. This agent was termed "sockeye salmon virus" (SSV) following further investigations confirming the viral etiology of the disease (Watson et al., 1954; Guenther et al., 1959). The virus was widely distributed among fish in hatcheries in Washington State, especially along the Columbia River.

In 1958, juvenile sockeye salmon at the Willamette River Hatchery in Oregon suffered an epizootic with high mortality. The etiological agent was isolated in cell culture and shown to be virulent for sockeye salmon but not for chinook salmon or rainbow trout (Salmo gairdneri) The virus replicated well in cell lines derived from sockeye salmon and steelhead trout but satisfactory replication in chinook salmon cells occured only after serial passage (Nims et al., 1970). This isolate was considered to be similar to the SSV strain because of the signs caused, host affected, and because fish released at hatcheries by either strain enter the Columbia River.

Ross et al. (1960) reported a filterable agent responsible for annually recurring mortalities in juvenile chinook salmon. The agent replicated in both steelhead trout and chinook salmon cell lines (Nims et. al., 1970), and was shown to be infective by the waterborne route for rainbow and steelhead trout as well as sockeye salmon (Wingfield and Chan, 1970). Adult steelhead and rainbow trout held at the same hatcheries as the affected juvenile chinook salmon were shown to be asymptomatic carriers of virus, as were adult chinook salmon returning to the hatcheries. Despite the presence of both chinook salmon and steelhead trout juveniles at hatcheries on the Sacramento and Feather Rivers, mortality was observed only in chinook salmon (Parisot and Pelnar, 1962; Wingfield and

Chan, 1970). Recently however, major losses of juvenile steelhead have occurred in the Sacramento River basin (W.H. Wingfield, California Department of Fish and Game, personal communication). The ability to cause natural mortality in chinook salmon suggested the term "Sacramento River chinook disease" (SRCD).

A virus was isolated by Amend et al. (1969) from rainbow trout and sockeye salmon reared in British Columbia hatcheries. Histopathological examination of the infected fish showed necrosis of the hematopoietic tissue of the kidney and spleen, leading to the name "infectious hematopoietic necrosis". An electron microscopy study of the British Columbia virus, the Oregon SSV and the SRCD virus showed similarly sized, bullet-shaped particles and other *in vitro* properties were found which were common to the rhabdovirus group. The pathology in fish and the cytopathic effect (CPE) in cell cultures were also similar (Amend and Chambers, 1970). The suggestion that the three agents were strains of the same virus, IHNV, has met with general acceptance. A serological comparison of the three strains showed identity by cross-neutralization of the Oregon SSV and British Columbia strains of IHNV. The strain from chinook salmon did cross neutralize with the other two but was less closely related (McCain et al., 1971).

Adult chinook salmon returning to the Elk River Hatchery in southern Oregon during the winter of 1975-76 were found to be IHNV carriers. The progeny of these fish suffered an IHNV epizootic. In 1978 another epizootic occurred in juvenile chinook at this hatchery. Juvenile steelhead at the same hatchery were unaffected (Mulcahy et al., 1980). Fendrick et al. (1982) found that this isolate had a slightly higher titer when assayed in a chinook salmon cell line than in a steelhead trout cell line.

In 1975, a series of IHNV isolations and viral epizootics began in the Metolius and Deschutes river systems in central Oregon (Mulcahy et al. 1980). Rainbow trout and landlocked sockeye (kokanee) salmon suffered mortality at the Wizard Falls Hatchery on the Metolius River in 1975. The source of the virus was thought to be either the water supply or infected kokanee salmon eggs from wild fish trapped in lakes at the headwaters of the Metolius and Deschutes rivers. Wild kokanee salmon spawning upstream from the hatchery were found to be asymptomatic virus carriers. Kokanee salmon in Suttle Lake, which drains into the Metolius River, were also found to be virus carriers (W.J. Groberg, Jr., Oregon Department of Fish and Wildlife, personal communication). This population had been used as an egg source for Wizard Falls Hatchery.

One month after the viral epizootic at Wizard Falls in 1975, mortality due to IHNV occurred in juvenile steelhead trout at Round Butte Hatchery (Mulcahy et al., 1980). This hatchery is situated below the confluence of the Metolius and Deschutes rivers. Virus has been isolated periodically both from adult steelhead trout used as an egg source, and from dying juvenile steelhead trout from the initial epizootic (1975-1982). Juvenile chinook salmon reared in the same hatchery have suffered no mortality, although virus has been detected in adult chinook salmon at Round Butte (Groberg and Fryer, 1983).

Grischowsky and Amend (1976) reported that IHNV was widely distributed in Alaskan sockeye salmon stocks and was responsible for large scale mortality in juvenile fish. No mortalities of chinook salmon caused by IHNV have been reported in the state; but virus has been isolated from asymptomatic adult chinook salmon (R. Grischowsky, Alaska Department of Fish and Game, personal communication). Virus from a sockeye salmon epizootic in Alaska was found to be avirulent for three stocks of chinook salmon (Wertheimer and Winton, 1982).

No mortalities of juvenile coho salmon (Oncorhynchus kisutch) caused by IHNV have been reported, and only one virus isolation from adult coho salmon has been made (W. H. Wingfield, California Department of Fish and Game, personal communication). Injection of juvenile coho salmon

with two IHNV strains has not produced mortality (Watson et al., 1954; Parisot and Pelnar, 1962). No losses have resulted from waterborne exposure to IHNV (Wingfield et al., 1970; Wingfield and Chan, 1970). The cell line derived from coho salmon has been found to be refractory to IHNV infection (Wingfield et al., 1970).

In their review of IHNV literature, Pilcher and Fryer (1980) concluded that isolates from sockeye salmon, chinook salmon and rainbow trout may exhibit different degrees of virulence and suggested that such differences deserved further investigation.

Variation in Susceptibility Within a Species

Watson et al. (1954) noted that mortalities occurred in four native sockeye salmon stocks in Washington State, suggesting that variation in susceptibility to IHNV between fish stocks was not sufficient to term any stock as resistant. Two California stocks of chinook salmon studied by Parisot and Pelnar (1962) appeared equally susceptible to IHNV. However, chinook salmon from Alaska were found to be more resistant to IHNV than a Columbia River chinook stock (Wertheimer and Winton 1982). Variation in susceptibility between offspring of different mating pairs of sockeye salmon indicates that resistance to IHNV can be genetically determined (Amend and Nelson, 1977). Certain sockeye salmon males were found to sire

IHNV-resistant progeny (McIntyre and Amend, 1978), although all fish used in a study of the heritability of IHNV resistance came from the same stock. Thus variation in IHNV susceptibility between stocks has been reported only in widely separated populations. Genetically-resistant individuals can exist within a population, and resistance can be genetically transmitted via sperm.

Viral Hemmorhagic Septicemia

The virus causing viral hemmorhagic septicemia (VHSV) is also a rhabdovirus causing mortality in juvenile salmonid fish. At present its distribution is limited to Europe (Pilcher and Fryer, 1980). Rainbow trout are the species most susceptible to the virus, while brown trout (*Salmo trutta*), coho salmon (de Kinkelin et al., 1974) and chinook salmon (Ord, 1975) are resistant.

Ord et al. (1976) compared the susceptibility of rainbow trout and coho salmon-rainbow trout hybrids to VHSV by waterborne infection. The hybrids were created by fertilizing rainbow trout eggs with coho salmon sperm. The susceptible rainbow trout suffered a 77% mortality but only 11% of the hybrid fish died. However, hybrids surviving infection were suspected of transmitting virus to uninfected rainbow trout.

In a manner resembling the differing host specificity of IHNV strains, a virus similar in morphology and *in*

vitro properties to VHSV was isolated from moribund brown trout. This agent was serologically unrelated to IHNV and distant from VHSV, suggesting that the new isolate was either a mutant VHSV or a second serotype (de Kinkelin and Le Berre, 1977).

Factors Determining Viral Host Range

The host range of a virus may be dependent on possession of the proper envelope protein. Love and Weiss (1974) showed that the growth of vesicular stomatitis virus (VSV) in cells containing an endogenous retrovirus genome yielded pseudotypes which possessed the genome of VSV and the envelope protein of the endogenous virus. This envelope conferred upon the VSV pseudotype the specific host range of the retrovirus, presumably by allowing adsorption to the new host cells.

A restriction in host range not due to failure of adsorption was demonstrated by Hsiung and Melnick (1957a). Five enteroviruses were analysed with respect to adsorption, multiplication and cytopathogenicity in susceptible and resistant cells from two monkey species. All viruses adsorbed well to both types of cells but in the resistant cells, the host-restricted viruses yielded few plaques. Growth curves of virus in liquid medium showed that little or no growth occurred in the resistant cells after a low inoculum. After a high inoculum, replication of the host-

restricted virus occurred, but with little cytopathic effect (CPE). Two of the viruses plaqued well in both types of cells, showing that the resistant cells are capable of producing CPE after enterovirus infection.

Comparison of the Structural Proteins of Infectious Hematopoietic Necrosis Virus Isolates

Two groups of investigators have characterized the structural proteins of IHNV by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. McAllister and Wagner (1975) found five structural proteins of molecular weight: 157,000, 72,000, 40,000, 25,000 and 20,000, designated L, G, N, M_1, M_2, respectively. Using similar methods, Hill et al. (1975) found five proteins of somewhat higher molecular weight.

Leong et al. (1981b) found that IHNV strains could be distinguished by slight differences in the molecular weight of the nucleocapsid protein, N. Differences could also be noted in the molecular weight of the G protein which is the envelope glycoprotein. In particular, the differences were pronounced between an isolate from chinook salmon at Coleman Hatchery in California and an isolate from steelhead trout at Round Butte. However, other chinook salmon isolates did not resemble the Coleman isolate, so the correlation between species of origin and the sizes of the N and G protein was not clear.

Infectious Hematopoietic Necrosis Virus Infection in Nature

In juvenile salmon and trout, mortality due to IHNV decreases with age. Wingfield and Chan (1970) found the highest susceptibility (80-100% mortality) was from the time of hatching to 10 days of age. Partial resistance appeared after 10 days (5-40% mortality) and complete resistance was attained at one month. In actual hatchery epizootics, losses can occur in sockeye salmon up to 12 months old (Watson et al., 1954) and in 3 month old chinook salmon (Wingfield and Chan 1970). The discrepancy in the period of susceptibility of experimental and hatchery fish may be due to the stress of crowding in the hatchery, or there may be a greater potential for horizontal transmission under hatchery conditions. Evidence for horizontal transmission of IHNV consists primarily of infections successfully initiated by placing uninfected juvenile fish downstream of infected fish (Rucker et al., 1953; Wingfield and Chan, 1970; Mulcahy et al., 1983). Fish have also been infected by placing moribund or dead fish in contact with healthy fish (Watson et al., 1954). Further evidence of horizontal transmission is the effectiveness of waterborne exposure to IHNV in initiating infection in the laboratory (Watson et al., 1954; Wingfield et al., 1970; Amend and Nelson, 1977).

Oral transmission of virus by feeding was probably responsible for the initiaton of IHNV epizootics in sockeye salmon in Washington and Oregon in the 1950's (Watson et al., 1954; Groberg et al., 1982). At that time, it was common practice to incorporate unpasteurized viscera of adult salmon, or salmon carcasses into the diet of the juvenile fish. Such viscera were shown to produce IHNV mortality after feeding to juvenile sockeye salmon (Watson et al., 1954). Cannibalism of diseased fish may also act to transmit the virus during an epizootic (Pilcher and Fryer, 1980).

Amend (1975) has shown that in trout surviving an IHNV epizootic, the infection enters a latent phase in which virus can no longer be isolated. However, at the time of sexual maturity, virus is shed in the reproductive fluids of the adults and can be found in the internal organs(Watson et al., 1954; Wingfield and Chan, 1970; Amend, 1975; Mulcahy et al., 1982). In chinook salmon populations in California, the proportion of IHNV positive fish increases as the spawning season progresses (Wingfield and Chan, 1970). The titer of virus in the internal organs of sockeye salmon increases with the onset of sexual maturity (Mulcahy et al., 1982). Only one report of mortality in adult fish has been attributed to IHNV (Mulcahy et al., 1980). It is generally agreed that adult salmonid fish infected with IHNV do not exhibit

pathological signs associated with IHNV (Watson et al., 1954, Wingfield and Chan, 1970; Pilcher and Fryer, 1980).

Adult carriers may act to maintain the virus by vertical transmission. Virus shed in semen and ovarian fluid is thought to result in transmission of the disease to the progeny (Parisot et al., 1965). Movement of eggs from hatcheries with a history of IHNV mortality to uncontaminated hatcheries has resulted in transfer of the disease (Parisot and Pelnar, 1962). At two locations, fish placed in the hatchery water supply remained free of IHNV while IHNV epizootics occurred in the hatchery, indicating that horizontal transmission was not responsible for initiating the epizootic. However, epizootics have occurred in the progeny of IHNV free adults at the same two locations (Wingfield and Chan, 1970). The relative importance of horizontal or vertical transmission may depend on the salmonid species, water supply, and physical arrangement of the hatchery involved. It seems clear that however it is initiated, an IHNV outbreak can be maintained and spread horizontally.

Effect of Temperature

Increased water temperature has been observed to reduce mortality due to IHNV in sockeye (Rucker et al., 1953; Watson et al., 1954) and chinook salmon (Ross et al., 1960). In both species, mortalities subsided as the

water temperature rose above 13°C.

Watson et al. (1954) held sockeye salmon infected by the waterborne route at temperatures from 4°C to 20°C. Fish held at 20°C had a 32% mortality while fish held at 4, 10 and 16°C had mortalities exceeding 85%. In a series of experiments, McAllister (1973) showed that the optimum temperature for production of IHNV mortality in sockeye salmon was 12°C, while temperatures of 17.8°C and 20.6°C reduced the mortality. The use of recirculated 15°C water was highly effective in preventing mortality in chinook salmon in controlled, large scale field trials (Wingfield and Chan, 1970).

Amend (1970) found that sockeye salmon exposed to virus had reduced mortality if the water temperature was increased from 12°C to 20°C within 24 h after infection and held at 20°C for at least 72 h before returning the temperature to 12°C. The rapid protective effect and the susceptibility of fish to reinfection immediately after heat treatment suggested that the protective effect was not mediated by the immune system. Heat-treated fish surviving infection were suspected of being virus carriers. Intraperitoneally injected sockeye salmon were also partially protected by 18°C water. Rainbow trout were protected by 17°C water temperature after intraperitoneal injection with IHNV (Amend, 1976).

One group of investigators found that mortality in rainbow trout was not reduced at water temperatures above 12°C (Hetrick et al., 1979). Using the waterborne route, fish were exposed to a much higher concentration than in previous laboratory tests, and the length of exposure was 24 h instead of the 30 min used by Amend (1970) and 60 min used by Watson et al. (1954). Such a level of IHNV may have rapidly overwhelmed the host and masked any protective effect of increased temperature.

The reason that increased temperature reduces mortality is unknown. The virus is routinely grown in cell culture at 18°C (Wingfield et al., 1969) and is stable in distilled water for 8 h at 23°C (Wingfield and Chan, 1970). One study comparing the growth rate of six isolates found that only the Coleman strain replicated more slowly at 18°C than at 15°C (Leong et al., 1981b).

Engleking and Leong (1981) established a persistently infected cell line which grew well in culture and produced virus at 22°C. Virus produced at 22°C had a higher titer when assayed at 16°C than at 22°C. A shift of the persistently infected cells down to 16°C resulted in loss of the virus-cell equilibrium and death of the cell culture. This persistently infected, temperature sensitive system was proposed as a model for the IHNV carrier state.

Plaque Size of Infectious Hematopoietic Necrosis Virus

Leong et al. (1981b) compared the mean plaque size of six IHNV isolates using methylcellulose overlay at 15°C. The Coleman isolate had a smaller mean plaque size, 0.317 mm, than the other isolates. This isolate was also found to have a slower rate of replication at 18 than at 15°C. An isolate from Alaska had the largest mean plaque size, 0.699 mm.

A wild type stock of IHNV was found to produce a mixture of large and small plaque sizes in cells treated with a polycation, polybrene (Leong et al., 1981a). On untreated cells, the large plaque type predominated. The enhanced infectivity of the small plaque type due to polybrene was observed when a tragacanth gum overlay was used, but not under an agarose overlay. The small plaque morphology was evidently due to a slower rate of replication at 16°C. Cloning of the mutant small plaque indicated that the phenotype was genetically controlled. However in a second publication, the small plaque was not found when the wild type stock virus was assayed using polybrene (Engleking and Leong, 1981). Small plaque types were found in virus released by a cell line persistently infected with IHNV and maintained at 22°C. Interference was noted when virus produced in the persistent cell line was titrated by endpoint dilution, and some evidence for the production of defective interfering particles was

found. The authors suggested that the small plaque phenotype was not due to temperature sensitive virus replication.

Plaque Size of Virus

Vogt et al. (1957) described a class of poliovirus mutants with a delayed (d) time of appearance and reduced efficiency of plaquing under a slightly acidic (pH 6.8) agar overlay, when compared to the wild type (d^+). The replication rate of the d and d^+ forms was the same in liquid medium. Thus the d characteristic was only shown under agar. Small plaque variants (s) of poliovirus which grew slowly even under alkaline conditions (7.5) were also found to be inheritable (Sabin 1956). The d and s mutations are independent: s^+d revertants were occasionally found in plaque-purified s populations. The effects of d and s are additive under an acidic agar overlay. Both d and s mutations correlate with loss of neuropathogenicity and the effects are again additive. These results were confirmed and extended by Hsiung and Melnick (1957b), who used the d marker to measure the extent of reversion in subjects fed live, attenuated poliovirus. However, these investigators indicated that the d characteristic was dependent on bicarbonate concentration rather than the pH of the overlay. Both Vogt et al. (1957) and Hsiung and Melnick (1957b) pointed out that the correlation of the d

marker to neurovirulence was a general one and that not all d^+ virus was pathogenic, suggesting that a third genetic factor such as host range might be involved. Vogt et al. (1957) speculated that viruses bearing the d characteristic might have an *in vivo* role, giving rise to latent infections which could later be activated by physiological changes in the host.

From a stock of vesicular exanthema of swine virus (VESV), McClain et al. (1958) purified two plaque types by cloning in swine kidney cells. The large plaque form (LP) of the the virus was pathogenic for swine but the small plaque form (SP) was virulent. In an *in vitro* study, McClain and Hackett (1959) found that the SP virus adsorbed to and multiplied more rapidly in swine kidney cells than the LP form. However, the SP virus was very cell associated, especially at pH 7. The LP virus was released from cells more rapidly at pH 7 and 8, and was more stable at these pH levels than the SP virus. The differences in plaque morphology were attributed to differences in the rate of release of virus and pH stability.

Wagner et al. (1963) cloned LP and SP forms of VSV from a stock culture. In chick embryo cells, only a slight difference in growth rate was noted, but in mouse L-929 (L) cells the CPE and yield of the SP form were substantially reduced. The two forms were antigenically

similar but not identical, and slight differences in buoyant density were detected. Both forms were capable of inducing interferon, but the SP variant was much more sensitive to interferon produced by L cells. Persistent infections of L cells with the SP virus could be established, from which only SP virus was produced, but no interferon was detected in the medium.

In a review of rhabdovirus genetics, Flamand (1980) states that SP variants of VSV are generally slower in replication rate and less virulent than the wild type LP virus. The comparative avirulence of SP mutants as a general characteristic of animal viruses was noted by Takemoto (1966) in a review of plaque mutants.

MATERIALS AND METHODS

Cell Lines

Four continuous cell lines derived from salmonid fish embryos by Fryer et al. (1965) were used in this study: chinook salmon (CHSE-214), steelhead trout (STE-137) and sockeye salmon (SSE-5) cells, all of which display epithelial morphology, and coho salmon (CSE-119) cells which are fibroblastic. Rainbow trout hepatoma cells (RTH-149) demonstrate epithelial morphology (Fryer et al., 1965) and were used in one experiment. These five cell lines have been characterized previously with respect to growth characteristics, chromosome number and susceptibility to fish viruses (Phillipon-Fried, 1980). Methods for routine cultivation of fish cells were described by Wolf and Quimby (1973).

The cell lines were grown in Eagle's minimum essential medium (MEM) with Earle's salts (Grand Island Biological Co.). The medium was supplemented with 5% fetal bovine serum (MEM-5), penicillin 100 I.U./ml, streptomycin 100 ug/ml (Grand Island Biological Co.) and the pH adjusted to 7.7-7.8 with 7.5% sodium bicarbonate. Cells were grown at 16^{o}C in plastic culture flasks (Corning Glass Works).

Viruses

Six IHNV isolates were used in this study (Table 1) to determine comparative virulence and infectivity for fish cell lines. All viruses were received from cooperating diagnostic laboratories as primary isolates or were collected as part of this study. Low passage isolates were used in order to minimize possible alterations in host specificity or loss of virulence due to extended cultivation in cells (Wingfield et al., 1970). Viruses were determined to be IHNV by neutralization with known anti-IHNV antiserum (American Fisheries Society: Fish Health Section, 1979). The Elk River (ER) and Feather River (FE) viruses were isolated from dead juvenile chinook salmon. The Trinity River (TR) and Alaska (AK) isolates were obtained from the ovarian fluid of spawning adult chinook salmon. An isolate from the ovarian fluid of adult steelhead trout at Round Butte was designated RBA and virus was obtained from diseased juveniles at the same location (RBJ).

Preparation of Virus Stocks

Stock viruses were grown by inoculating newly confluent monolayers of CHSE-214 cells in 150 cm^2 flasks. Viruses received as primary isolates were filtered (0.2 um pore diameter), diluted 1:5 in MEM-0, and 1 ml was added to each flask. Other stocks of virus were seeded at a

Table 1. Infectious hematopoietic necrosis virus isolates used in this study.

Virus Isolate	Host Isolated From	Location	No. of Cell Line Passages
ER	Chinook juvenile[a]	Elk River, OR	2-3
FE	Chinook juvenile	Feather River, CA	2-3
TR	Chinook adult[b]	Trinity River, CA	2-3
AK	Chinook adult	Mendenhall, AK	2-3
RBA	Steelhead adult	Round Butte, OR	0[c]-3
RBJ	Steelhead juvenile	Round Butte, OR	0[d]-2

[a]All IHNV isolates from juveniles used in this study were from diseased fish.

[b]All IHNV isolates from adult fish used in this study were from carrier fish.

[c]Sterile filtered (0.45µm pore diameter) ovarian fluid.

[d]Dead fish were homogenized in MEM-0, centrifuged, and a filtrate (0.45µm pore diameter) was 0-pass IHNV.

multiplicity of infection of 0.01. After adsorption for 1 h at 16°, 30 ml of MEM-5 were added and the flasks sealed. Virus was harvested after 8-10 days, when CPE was complete. The medium was centrifuged at 2000 x g for 10 min, aliquoted and frozen at -70°C. The titer of 2-3 passage IHNV ranged from $10^{5.5}$-$10^{7.1}$ tissue culture infective doses ($TCID_{50}$/ml). Unpassaged RBJ was prepared by homogenizing juvenile mortalities in MEM without serum (MEM-0), and centrifuging the debris at 2000 x g for 10 min. The filtrate (0.45 μm pore diameter) was stored at -70°C.

$TCID_{50}$ Assay

The $TCID_{50}$ assay was used to quantify virus in ovarian fluids and supernatants from IHNV infected cells. Cells were inoculated into 96-well microplates (Flow Laboratories, Inc.) on the day previous to each $TCID_{50}$ assay. Each well received 0.1 ml of MEM-5 containing approximately $5x10^5$ cells/ml. Tenfold dilutions of fluids containing virus were made in MEM-0. An 0.1 ml aliquot of each dilution of virus was placed in each of the 8 wells in one row of the microplate. Plates were sealed with mylar film and incubated at 16°C for 10 to 14 days. The number of wells showing CPE were recorded and the virus concentration in $TCID_{50}$/ml was calculated by the method of Reed and Muench (1938).

Monolayer Plaque Assay

Preparation of Monolayers

The monolayer plaque assay (Wolf and Quimby, 1973) was used to determine efficiency of plaquing and plaque size, and to purify plaque size variants. Cells grown in 150 cm^2 flasks were pooled in a common container before seeding into 25 cm^2 culture flasks (Corning Glass Works) such that 90-95% confluency was attained after two days.

Efficiency of Plaquing

Following removal of the growth medium, cell monolayers were washed three times with 1 ml of MEM-0 prior to adding 0.1 ml of virus diluted in MEM-0. Virus was inoculated into replicate flasks using a micropipette to ensure accurate delivery. Virus was adsorbed for 1 h at 16^{o}C on a rocking platform (Bellco Co.). Unadsorbed virus was removed with three washes of MEM-0 and 5 ml of MEM-5 containing 1% methylcellulose (Fisher Scientific Co.) were added to each flask. The number of IHNV plaques has been found to be greater under methylcellulose overlay than agarose overlay (Burke and Mulcahy, 1980). The flasks were incubated at 16^{o}C for 10 days, after which the monolayers were fixed with formalin (37%) for 30 min and stained with 1% crystal violet for 1 min. The concentration of virus was recorded as plaque forming

units per ml (PFU/ml) after determining counts in replicate flasks containing 20-300 plaques.

Plaque Size Determination

Growth medium was removed from CHSE-214 cell monolayers and 0.2 ml of virus diluted in MEM-0 added to replicate flasks. Virus was adsorbed for 1 h at 16^{o}C on a rocking platform. Fluid was aspirated before adding 8 ml of MEM-5 with 0.8% agarose (Seakem Inc.). Plaques produced by IHNV under agarose are larger than when methylcellulose overlay is used (Burke and Mulcahy, 1980). The larger plaque size permits a greater accuracy of measurement. Flasks were incubated in an inverted position. Monolayers were fixed with formalin (37%) and stained with crystal violet. Plaque sizes were measured to the nearest 0.1 mm with a dial micrometer and colony counter.

Some variation was observed in the mean plaque size of a virus stock determined in different experiments. This may have resulted from slight variation in the physiological condition of the CHSE-214 cells. To minimize this variation, comparisons were made only between plaque assays performed on the same day. The point of division between large and small plaques was determined by discontinuities in plaque size-frequency histograms plotted at the end of each experiment.

Purification of Plaque Size Variants

Monolayered CHSE-214 cells were inoculated with virus to produce about 20 plaques per flask. After 10 days of incubation at 16°C, well separated small (<1 mm) and large (>2mm) plaques were selected. Agar plugs were removed and placed into monolayer cultures of CHSE-214 cells in 24-well plates (Flow Laboratories, Inc.). Virus was harvested after complete lysis (about 6-7 days), centrifuged at 2000 x g for 10 min and frozen at -70°C. This plaque purification procedure was repeated four times. The lysate from the fourth cycle was used to grow stock virus in CHSE-214 cells.

Source of Juvenile Salmonids Used in Virulence Testing

Juvenile fish, eggs, and semen were obtained from four species of salmonid fish: chinook, sockeye (kokanee) and coho salmon, and steelhead and rainbow trout. Fish were provided by the Oregon Department of Fish and Wildlife from selected hatcheries (Table 2). The susceptibility of experimental infected juvenile salmonid fish declines rapidly with age (Wingfield and Chan, 1970); accordingly, fish were transported from the hatchery to the Oregon State University Fish Disease Laboratory as soon as they could be handled. Fish were held for two days to recover from transport stress prior to virus exposure. The difference in the weight at testing of the

Table 2. Sources of fish used to determine virulence of virus isolates.

Name	Race	Source	Size at Testing (gm)
Sockeye salmon	Kokanee	Wizard Falls Hatchery Camp Sherman, OR	0.18
Steelhead trout	Summer	Round Butte Hatchery Madras, OR	0.23
Steelhead trout	Summer	Oak Springs Hatchery Maupin, OR	0.26
Rainbow trout	Fall	Oak Springs Hatchery Maupin, OR	0.21
Chinook salmon	Spring	Round Butte Hatchery Madras, OR	0.41
Chinook salmon	Fall	Trask River Hatchery Tillamook, OR	0.56
Chinook salmon	Fall	Elk River Hatchery Port Orford, OR	0.57
Coho salmon	-	Sandy River Hatchery Sandy, OR	0.35 0.45 0.95
Coho salmon	-	Salmon River Hatchery Otis, OR	0.46

different species (Table 2) primarily reflected the difference in the size of eggs produced by the species, which in turn determines size at hatching. Except for one experiment in which the increase in resistance with size and age was investigated, fish were exposed to virus during the last stages of yolk absorbtion, when active feeding begins. All procedures involving live fish or fish eggs were carried out at 12°C in fish pathogen-free well water.

Hybridization of Coho Salmon and Rainbow Trout

Coho salmon eggs from three fish at the Sandy River Hatchery were pooled. Rainbow trout eggs were collected from a fish at the Oak Springs Hatchery. Semen was collected from 10 males and pooled at each location. Gametes were transported under refrigeration and without the addition of fluid to the Fish Disease Laboratory. Fertilization was accomplished by gently mixing 10 ml semen per 1000 eggs. Additional water was added for 1 min before distributing eggs into incubation trays. Eggs were incubated in the dark. Dead eggs and yolk-sac fry were removed and recorded daily from seven days after fertilization until active feeding began.

Hybrids created by fertilizing 4000 rainbow trout eggs with coho salmon sperm underwent greater cumulative mortality (98%) during the incubation period than rainbow

trout (35%) and coho salmon (40%) produced from the same gamete pools. To verify that the coho salmon-rainbow trout cross produced true interspecific hybrids, the liver isoenzymes of the hybrid and parental species were compared by electrophoresis. The electrophoresis was performed by the laboratory of Dr. G.A.E Gall, Department of Animal Science, University of California, Davis. In each enzyme system studied, the 10 hybrid fish examined displayed the isoenzyme bands of both parental species, demonstrating that the coho salmon-rainbow trout cross produced true F_1 hybrids.

Exposure of Juvenile Salmon and Trout to Virus

Waterborne virus exposures were used to simulate natural horizontal transmission. Virus isolates to be compared for virulence were tested simultaneously against a given fish stock. Due to the seasonal availability of juvenile salmonid fish, the virus exposures took place over a period of 18 months. Retitration of virus stocks one year after preparation showed no loss in titer during storage at -70°C.

Frozen virus twice-passaged in CHSE-214 cells was thawed and diluted in MEM-5. Dilutions of virus were prepared such that concentrations of 10^1-10^5 $TCID_{50}$/ml were achieved when virus was added to glass jars containing 750 ml water. Each jar contained 20 chinook or

coho salmon or 30 of the smaller trout or kokanee salmon. Due to the small number of hybrid fish available, 10 fish were tested at each viral dilution. Each experiment included control fish exposed to MEM-5 with no virus. Temperature during the 12 h exposure was controlled by partially submerging the jars in flowing 12^{o} water. Compressed air was bubbled through an airstone in each jar to provide oxygenation. After a 12 h exposure, the contents of each jar were placed into a 68 L fiberglass aquarium receiving single-pass well water flowing at a rate of 1 L/min. Fish were fed twice daily with the appropriate size of Oregon Moist Pellet diet containing 1% oxytetracycline (Pfizer) to prevent bacterial infection. Fish were observed for symptoms of IHNV infection for 14 days after exposure. Dead or moribund fish were removed daily and frozen at -20^{o}C unless immediately processed to recover virus.

Recovery of Virus From Dead Fish

A pooled group of five fresh or frozen fish was assayed to confirm the presence of IHNV each time a fish stock (Table 2) was exposed to a virus isolate (Table 1). Dead coho salmon were individually examined for virus. Fish were homogenized in MEM-0 at a 1:10 dilution (w/v) using a Stomacher (Dynatech Laboratories Inc.). The homogenate was centrifuged at 2000 x g for 10 min. The

supernatant was filtered (0.45 μm pore size) and 0.5 ml was added to 2.0 ml of an antibiotic solution containing 1000 IU/ml penicillin, 1000 ug/ml streptomycin, 0.25 mg/ml gentamycin and 5 mg/ml amphotericin B (Gibco). After overnight incubation at 4°C, 0.25 ml of the virus-antibiotic mixtures were added to monolayers of CHSE-214 cells prepared in 24 well plates. The samples from coho salmon were inoculated onto SSE-5 cells and CHSE-214 cells. Plates were sealed with mylar film and incubated at 16°C. Cells were observed over 10 days for typical IHNV CPE. After 10 days, 0.01 ml of medium was removed from each primary isolation culture and added to a new cell culture prepared in a 24 well plate. This was done to confirm the presence or absence of IHNV based on the appearance of characteristic IHNV CPE after 10 days of incubation at 16°C.

EXPERIMENTAL RESULTS

Determination of Susceptibility of Four Salmonid Species to Virus Isolates

Chinook, coho and sockeye salmon, and steelhead trout were exposed to twice-passed ER, TR, AK and RBA to determine if susceptibility to viral isolates differed between species. Three stocks of chinook salmon were exposed to determine if intraspecific variation in susceptibility could be detected among Oregon chinook salmon (Table 3). The 50% lethal dose (LD_{50}) was calculated according to the method of Reed and Muench (1938). Mortalities of chinook salmon after IHNV exposure were generally lower than mortalities in sockeye salmon and steelhead trout. Due to insufficient mortality, a LD_{50} could not be calculated for some virus isolates in chinook salmon, so the less precise 25% lethal dose (LD_{25}) was calculated.

Comparisons were made between the four LD_{50} values determined in a single fish stock to determine the relative virulence of the isolates. Comparisons of absolute LD_{50} values between different fish species and stocks were less reliable because of unequal sizes and because the various fish stocks were exposed at different times. Differences of less than 0.5 between the log LD_{50} of virus isolates were regarded to indicate similar virulence. The

Table 3. Comparison of virulence of four infectious hematopoietic necrosis virus isolates for selected Oregon salmonid stocks.

Virus	Log LD_{50} in $TCID_{50}$/ml		Log LD_{25} in $TCID_{50}$/ml			
	Wizard Falls sockeye 0.18 gm[a]	Round Butte steelhead 0.23 gm	Round Butte chinook 0.42 gm	Trask River chinook 0.54 gm	Elk River chinook 0.57 gm	Sandy River coho 0.35
AK	2.0	2.7	4.8	4.3	>5.0	3.6
ER	2.9	3.5	4.0	3.2	4.2	4.5
RBA	2.2	2.7	5.0	4.8	>5.0	4.2
TR	2.4	2.8	3.3	2.3	2.1	3.7

[a]Average size of fish at time of exposure.

value of 0.5 $\log_{10}$ was chosen after consideration of the data in Table 3, possible error due to biological variation in the fish, and the objective of fisheries management to avoid failing to detect differences in virulence. In a previous experiment, duplicate exposures of groups of 30 fish to tenfold dilutions of IHNV resulted in log LD_{50} values which differed by less than 0.1, therefore differences greater than 0.5 $\log_{10}$ are probably biologically meaningful. Probit transformations (Finney, 1952) were used to plot the percent mortality against the $\log_{10}$ virus concentration. Whenever curves could be fitted to the data, the 95% confidence intervals of the LD_{50} or LD_{25} were calculated. The results of these calculations tended to confirm the validity of using 0.5 $\log_{10}$ to differentiate the virulence of isolates.

The various isolates possessed different degrees of virulence in chinook salmon (Table 3). In all three stocks examined, the TR appeared to be the most virulent, followed by ER. The AK and RBA isolates had similar LD_{50} values in Round Butte and Elk River chinook salmon, but AK seemed to be more virulent than RBA in Trask River chinook salmon. Because all three chinook stocks were more susceptible to TR and ER than RBA and AK, an unidentified but virus isolate-specific factor may determine the outcome of exposure to virus.

Although belonging to different genera, sockeye salmon and steelhead trout exhibited a similar pattern of susceptibility to the four isolates. The LD_{50}'s of AK, RBA and TR were similar to each other, and ER had the highest LD_{50} in both species. Because AK and RBA appeared to be less pathogenic than TR in chinook salmon, but seemed to be as virulent or more virulent than TR in sockeye salmon and steelhead trout, an undetermined host factor may also determine the outcome of exposure to IHNV.

Elk River chinook salmon were exposed to FE. This isolate had an LD_{50} of $10^{3.5}$ $TCID_{50}$/ml for chinook salmon and $10^{4.3}$ $TCID_{50}$/ml for steelhead trout. These results suggested that FE was somewhat less virulent than TR (which had a LD_{50} of $10^{2.8}$ in Elk River chinook) but more virulent than ER, AK and RBA in chinook salmon.

When the LD_{25} values for all four virus isolates were compared between the three chinook salmon stocks, Round Butte and Elk River fish appeared to be less susceptible to IHNV. Because the LD_{25} values are less precise than LD_{50} values, and because the fish could not be exposed simultaneously, intraspecific variation in susceptibility could not be conclusively demonstrated.

Twenty Trask River chinook salmon exposed to 10^3 $TCID_{50}$/ml AK experienced no mortality. At the conclusion of the 14 day observation period, these fish were challenged with 10^5 $TCID_{50}$/ml of FE for 12 h. Twenty fish

previously exposed to MEM-5 were similarly challenged with FE. A third untreated (control) group was exposed to MEM-5 only. During a 14 day observation period, three fish died in the group previously exposed to AK, while nine fish died in the MEM-5 treated group after virus challenge. Typical symptoms of IHNV infection were observed and virus was recovered from both virus challenged groups. No losses occurred among the control fish. In this experiment, AK appeared to act as an attenuated virus with a protective effect in chinook salmon after only two passages in cell culture.

Virus was recovered from dead or dying fish in each combination of virus isolate and fish stock, except in Elk River chinook salmon, where no fish died after exposure to AK and RBA. Neutralization with anti-IHNV antiserum confirmed that AK, RBA, ER and TR recovered from experimentally infected sockeye salmon mortalities was IHNV. No virus was recovered from similarly sampled control fish in each experiment. Mortality in control fish did not exceed 5% in any experiment.

Mortality was observed in juvenile coho salmon from Sandy River Hatchery exposed to AK, ER, RBA and TR. The losses were more severe in fish exposed to AK and TR than in the other two isolates (Table 3). The LD_{50} ($10^{4.5}$) and LD_{25} ($10^{3.7}$) of the highly virulent TR were apparently higher in coho salmon than in the other three species

tested, suggesting that this species is more resistant to IHNV.

The first mortalities in coho salmon exposed to IHNV occurred at four days, and mortality declined after day 11, which was similar to what was observed during experiments with steelhead trout, and sockeye and chinook salmon. Typical signs of IHNV infection were not common in coho salmon, although darkened coloration and hemorrhage in the head area were noted in a few fish. Fungus was noted on the head, gills and body surfaces of several moribund fish. Twenty control coho salmon exposed to MEM-5 and held under identical conditions experienced no mortality.

Dead coho salmon were individually examined for IHNV, using both CHSE-214 and SSE-5 cells. Most dead fish were examined immediately but a few were tested for virus after frozen storage. Virus was recovered from about 50% of the dead fish collected after exposure to each of the four virus isolates (Table 4).

Histopathological examination of five virus-exposed, moribund coho salmon was performed by W.T. Yasutake, National Fisheries Research Center, Seattle. Subtle cytopathological changes were observed in the hematopoietic tissue of the kidney of these fish, usually involving one to several cells. The cells resembled necrobiotic bodies, which are pathognomonic of IHNV infection (Yasutake,

Table 4. Number of deaths and percent recovery of virus from Sandy River coho salmon[a] exposed to four infectious hematopoietic necrosis virus isolates.

Virus	No. Deaths/20 fish					No. Positive for Virus / No. Sampled	Percent Positive for Virus
	10^5[b]	10^4	10^3	10^2	10^1		
AK	12(3)[c]	8(7)	3(1)	0	0	11/21	52
ER	5(2)	0	4(3)	0	0	5/9	56
RBA	6(3)	6(1)	1(1)	0	0	5/10	50
TR	11(9)	5(0)	1(0)	3(2)	2(1)	12/22	55

[a]Fish were tested at an average weight of 0.35 gm. Twenty Sandy River coho salmon were controls and exposed to the diluent used to suspend virus. No mortality was observed in this group.

[b]Concentration of IHNV in $TCID_{50}$ per ml used in 12 h waterborne exposure.

[c]Values in parentheses indicate the number of dead fish from which virus was isolated.

1978). Degenerative changes found in the pancreatic acinar cells were also probably related to IHNV infection.

Fifteen dead coho salmon from the four virus-exposed groups were examined for bacterial pathogens. Kidney tissue was streaked on trypticase soy agar (Difco) to detect Gram-negative rods and cytophaga agar to detect Cytophaga psychrophilia, the causative agent of low-temperature disease of coho salmon (Pacha, 1968). The area posterior to the dorsal fin was also cultured on cytophaga agar. The number and type of colonies which appeared on the trypticase soy agar plates indicated that the mortalities were probably not due to a Gram-negative bacteremia. No low-temperature disease organisms were isolated on cytophaga agar.

The exposure of coho salmon to IHNV was repeated with larger coho salmon from Sandy River Hatchery (1.05 gm and 0.45 gm) and Salmon River Hatchery (0.45gm). Fish were exposed to third-passage AK and TR virus. No mortality was observed in these fish. Dark coloration and lethargy were observed in two of the 0.45 gm Sandy River fish at 8-11 days after exposure to 10^5 $TCID_{50}$/ml of TR; however, these fish resumed normal feeding by 14 days. A fish exposed to 10^5 $TCID_{50}$/ml AK was sampled after exhibiting similar signs, but no virus was recovered. However, virus was recovered from 2 of 10 asymptomatic 0.45 gm Sandy River coho sampled at 11 days after exposure to 10^4

$TCID_{50}$/ml TR. Virus was also recovered from 2 of 10 surviving 0.45 gm Sandy River fish exposed to 10^5 $TCID_{50}$/ml TR. These four isolations were confirmed to be IHNV by neutralization with anti-IHNV antiserum. No virus was recovered from the 1.05 gm Sandy River coho, any fish from Salmon River, or fish exposed to AK. A low incidence of virus infection without mortality was demonstrated in these experiments.

All stocks of fish shown in (Table 3) were susceptible to TR. The results of exposure to this virus were compared to determine if mortality was dose-dependent (Table 5). A regression function between the $\log_{10}$ of the virus concentration and the percent mortality was determined by the method of least squares. The Pearson correlation coefficient, which expresses the degree of relatedness between the $\log_{10}$ virus concentration and the percent mortality was determined. The least correlation was found in coho salmon. The F statistic for the hypothesis that the slope of the regression (b_1) was equal to zero was calculated. Confidence limits for the F statistic were approximately equal to or greater than 90% in all cases, suggesting dose dependent mortality. Mortality did not appear to be dose dependent if a stock was resistant to an isolate. For example, when Round Butte chinook were exposed to RBA, the percent dead at virus concentrations of 10^5-10^1 were 15, 10, 0, 0, and 5% respectively.

Table 5. Dose dependence of cumulative mortality 14 days postexposure to Trinity River infectious hematopoietic necrosis virus.

Fish Stock	Percent Dead at Each Virus Concentration						r[c]	C.L.[d]
	10^5[a]	10^4	10^3	10^2	10^1	0[b]		
Wizard Falls sockeye salmon	100	97	73	33	7	0	.97	99
Round Butte steelhead trout	83	73	57	23	0	3	.98	99
Round Butte chinook salmon	75	30	16	3	0	0	.92	95
Trask River chinook salmon	100	80	50	20	5	0	.99	99
Elk River chinook salmon	95	70	65	35	0	0	.97	99
Sandy River coho salmon	55	25	5	15	10	0	.79	90

[a]Virus concentration in $TCID_{50}$ per ml used in 12 h exposure.

[b]Control fish were exposed to the diluent, MEM-5.

[c]Pearson correlation coefficient of relatedness between $\log_{10}$ virus per ml and the percent mortality.

[d]Confidence level of F test for b_1 (slope of regression line) = 0.

Resistance of Coho Salmon-Rainbow Trout Hybrids to Virus

Groups of 10 coho salmon, rainbow trout, or coho-rainbow hybrids were exposed to TR at concentrations of 10^3-10^5 $TCID_{50}$/ml. Coho salmon and coho-rainbow hybrids were more resistant than rainbow trout, even though the hybrids were of equivalent size to the rainbow trout (Table 6). Probit analysis of the results of exposure of rainbow trout to virus indicated that the 95% confidence interval about the LD_{50} was + 0.78. Therefore the difference in susceptibility between rainbow trout and the other two varieties of fish appeared to be significant. Virus was recovered from pooled dead hybrids and pooled dead rainbow trout. Coho salmon were individually sampled. Virus was recovered from 1 of 5 dead or moribund coho salmon. Virus was also recovered from surviving hybrids and coho salmon 14 days after exposure. Control groups of coho salmon, rainbow trout and coho-rainbow hybrids were exposed to MEM-5. The only control mortality was a hybrid fish and no virus was found in this animal. Resistance appeared to be inherited as a dominant Mendelian trait. The sex of the hybrids could not be determined due to the small size and the need to sample for virus, so it is not known whether IHNV resistance was sex-linked or autosomal.

Table 6. Resistance of rainbow trout, coho salmon and rainbow trout-coho salmon hybrids to infectious hematopoietic necrosis virus.

Species	Size (gm)	No. of Mortalities/10 fish 10^5[a]	10^4	10^3	0[b]	LD_{50} in $TCID_{50}$/ml
Rainbow trout	0.21	8	6	0	0	$10^{4.0}$
Coho salmon	0.35	6	0	0	0	$10^{4.8}$
Hybrid	0.19	3	0	1	1	$>10^{5.0}$

[a]Concentration of Trinity IHNV used in 12 hr waterborne exposure.

[b]Control fish were exposed to the virus diluent, MEM-5.

Infectivity of Virus Isolates in Salmonid Cell Lines

Infective Dose Determination in Cell Lines

Virus isolates grown in CHSE-214 cells were titered by the $TCID_{50}$ assay in selected salmonid cell lines to determine if the titer of a virus isolate depended on the species from which the cell line was derived. Third-passage FE, ER and RBA were titered in CHSE-214, SSE-5 and STE-137 cells. The final examination of the wells of each plate for CPE was at 14 days. No increase in the number of positive wells was observed after 12 days. Experiments indicated that duplicate $TCID_{50}$ assays conducted on the same day resulted in titers which differed by less than 0.1 $\log_{10}$. Titers of an isolate in different cell lines which differed by 0.5 $\log_{10}$ or less were considered to be similar. Up to one $\log_{10}$ differences between the titers of an isolate on different cell lines were found (Table 7). The FE and ER isolates displayed a much higher titer on CHSE-214 cells than on STE-137 cells. This result may have been a reflection of the virulence of these two isolates for chinook salmon. The titer of RBA was highest on SSE-5 cells, but was similar on both CHSE-214 and STE-137 cells.

A second series of titrations was carried out using the AK and TR isolates passaged three times in CHSE-214 cells. Virus was titered on CHSE-214, SSE-5, STE-137,

Table 7. Infective dose determination of three virus isolates in cell lines derived from chinook and sockeye salmon, and steelhead trout.

	Virus Isolates		
Cell Lines	FE	ER	RBA
CHSE-214	$10^{7.2}$[a]	$10^{6.7}$	$10^{5.6}$
SSE-5	$10^{6.3}$	$10^{6.6}$	$10^{6.1}$
STE-137	$10^{5.7}$	$10^{5.6}$	$10^{5.7}$

[a]Virus titer expressed in $TCID_{50}$ units per ml of undiluted stock virus.

RTH-149 and CSE-119 cells. The coho salmon cell line was included because of the mortality observed when coho were exposed to IHNV. The titer of AK was more than 10 times higher in SSE-5 cells than in CHSE-214 cells (Table 8). Although AK was isolated from chinook salmon and grown in CHSE-214 cells, the titer was highest in cells derived from the host species (sockeye salmon) most affected at the site where AK was isolated. Thus the results of the $TCID_{50}$ assays were not primarily determined by the cell line used to prepare the virus stocks. The titer of TR was highest in the cells (CHSE-214) of the affected host (chinook salmon), but the titers in CHSE-214, SSE-5, RTH-149 and STE-137 cells were similar (Table 8). The similarity between the titers may be related to the high virulence of TR for juvenile salmonids of the three species from which the cell lines were derived. The titer of AK was identical on the cell lines derived from the two types of Salmo gairdneri, RTH-149 and STE-137. The titer of TR was also similar on these two cell lines, further suggesting that the species of origin may be an important factor in determining the susceptibility of a given cell line to a virus.

Titers of AK and TR were much lower in CSE-119 cells than in the other four cell lines (Table 8). These cells were derived from a species at least partially resistant to IHNV. The CPE in the CSE-119 cells was similar to the

Table 8. Infective dose determinations of two virus isolates in cell lines derived from chinook, coho and sockeye salmon and rainbow and steelhead trout.

Cell Line	AK Titer[a]	TR Titer
CHSE-214	$10^{6.5}$	$10^{6.8}$
SSE-5	$10^{7.6}$	$10^{6.6}$
STE-137	$10^{6.0}$	$10^{6.3}$
RTH-149	$10^{6.0}$	$10^{6.5}$
CSE-119	$10^{4.4}$	$10^{3.6}$

[a]Expressed as $TCID_{50}$ units per ml of undiluted stock virus.

CPE in the other cell lines, with rounded-up and balloon-shaped cells (Figure 1), but appeared two to four days later and often did not proceed to completion.

Efficiency of Plaquing of Virus in Different Cell Lines

The efficiency of plaquing (EOP) of FE, ER and RBA was compared on CHSE-214, SSE-5 and STE-137 cells. The virus isolates had been passed three times in CHSE-214 cells. The virus titers displayed in Table 9 are an average determined from six replicate flasks. The most direct comparison could be made between the titers of a single isolate in the three cell lines, because all 18 flasks were inoculated from the same dilution tube.

The titer of each isolate was dependent on the cell line used. Bartlett's test was performed to determine the homogeneity of the variance of the titer of each isolate on the three cell lines. The variance could not be shown to be significantly different at a 95% confidence level, so a pooled variance was used in further statistical comparison of the titers of each isolate. An analysis of variance showed that significant variation with $F>F_{.99}$ (df=2,15) existed between the titer of each isolate on the three cell lines. The titer of RBA was significantly lower ($P>.95$) by t test on CHSE-214 cells than SSE-5 and STE-137 cells, in agreement with the low virulence of this isolate for chinook salmon and the high virulence for

Figure 1. The round or balloon-shaped cells (arrow) form a focus of cytopathic effect in a cell line derived from coho salmon embryos (CSE-119). The CSE-119 cells were infected with infectious hematopoietic necrosis virus.

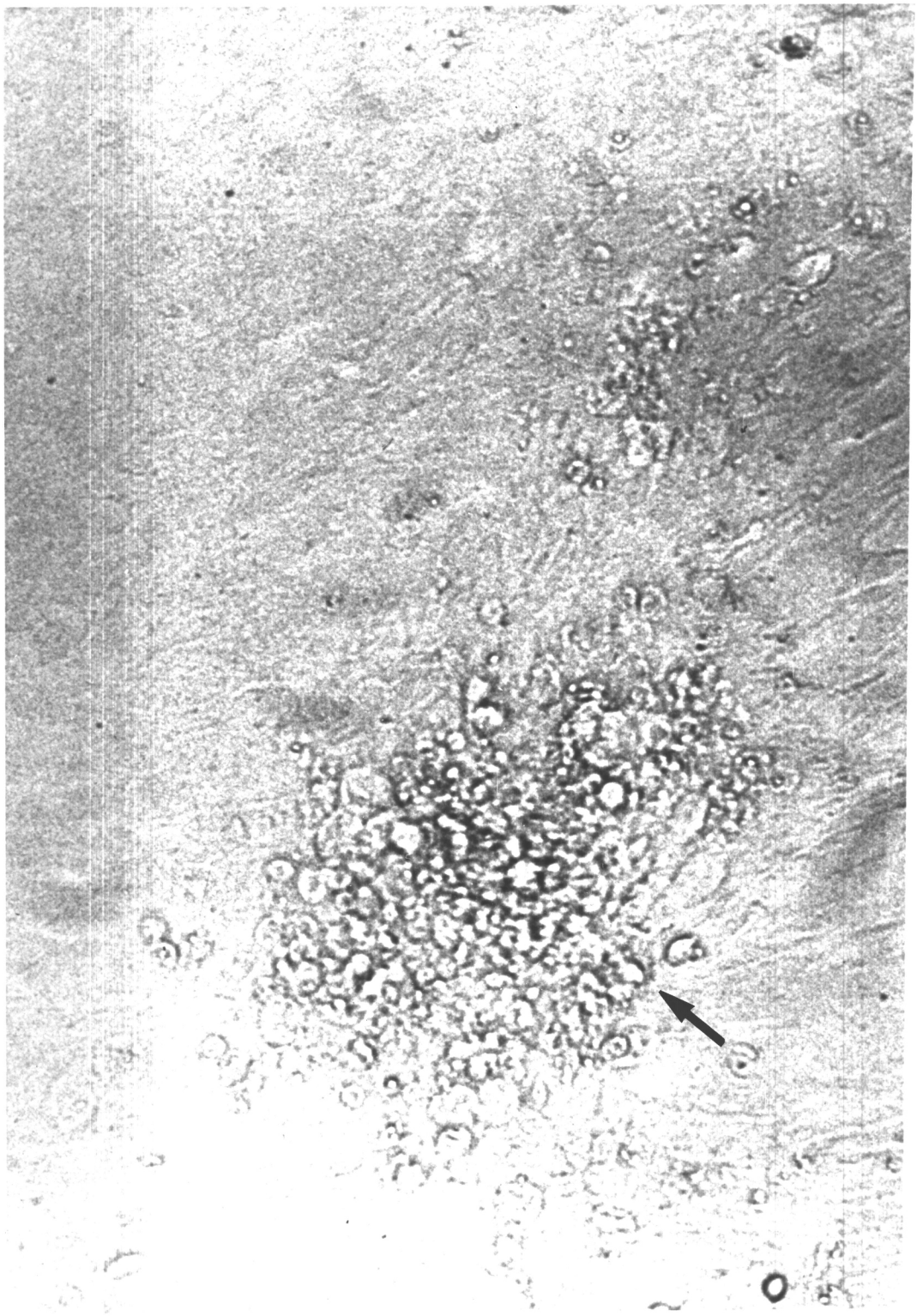

Figure 1.

Table 9. Plaque assay titer of three infectious hematopoietic necrosis virus isolates in cell lines derived from chinook and sockeye salmon, and steelhead trout.

Cell Line	Virus Isolate		
	RBA	ER	FE
CHSE-214	2.0×10^6 [a]	8.5×10^6	9.7×10^6
	s=0.4 [b]	s=2.0	s=2.1
STE-137	3.5×10^6	1.4×10^6	3.6×10^6
	s=0.8	s=0.4	s=0.9
	(1:1.75) [c]	(1:0.16)	(1:0.37)
SSE-5	4.5×10^6	7.1×10^6	10.2×10^6
	s=0.5	s=0.9	s=2.0
	(1:2.25)	(1:0.84)	(1:1.05)

[a] Virus titer in PFU per ml averaged from six replicate flasks.

[b] Standard deviation x 10^{-6} of titers calculated from six replicate flasks.

[c] Ratio of titer in CHSE-214 cells:titer in STE-137 SSE-5 cells.

steelhead trout. The titer of ER was significantly higher on CHSE-214 and SSE-5 cells than STE-137 cells ($P>.999$). A similar result was obtained for the FE isolate. Thus, the two isolates reported to cause epizootic mortality in chinook salmon but not steelhead trout displayed a higher EOP in chinook salmon cells than steelhead trout cells. High titers of all three isolates were obtained in SSE-5 cells.

The EOP assay was performed with two additional isolates, AK and TR, to test if the relative titers on SSE-5 and CHSE-214 cells corresponded to the virulence of the isolates for chinook salmon. The five isolates were also plaqued on CSE-119 cells, but no clearly definable plaques were observed. The titers in Table 10 were determined using an average of three replicate flasks. The ratio of the titers in CHSE-214 and SSE-5 cells was between 1:1 and 1:13 for the three isolates virulent for chinook salmon. The two isolates avirulent for chinook salmon, AK and RBA, had SSE-5/CHSE-214 titer ratios of 1:2.8 and 1:7.4 respectively, indicating that these two isolates had a low EOP in CHSE-214 cells. Both the $TCID_{50}$ and EOP comparisons suggested similarity between the comparative virulence and viral infectivity for cell lines derived from the host species.

Table 10. Plaque assay titer of five infectious hematopoietic necrosis virus isolates in cell lines derived from chinook and sockeye salmon.

	Virus Isolates				
Cell Lines	AK	RBA	ER	TR	FE
CHSE-214	1.2×10^7 [a]	8.0×10^5	5.3×10^6	6.2×10^6	8.7×10^6
SSE-5	3.3×10^7	5.9×10^6	5.1×10^6	8.2×10^6	1.0×10^7
	(1:2.8) [b]	(1:7.4)	(1:0.96)	(1:1.3)	(1:1.2)

[a] Virus titer in PFU per ml averaged from three replicate flasks.

[b] Ratio of titer in CHSE-214 cells:titer in SSE-5 cells.

Multicyclic Growth Curve of Virus in Chinook and Coho Salmon Cell Lines

An experiment was conducted to confirm the replication of IHNV in CSE-119 cells which had been indicated by the appearance of CPE in the $TCID_{50}$ assays. Approximately 10^4 $TCID_{50}$ of TR in 0.2 ml was used to infect two day old monolayers of CHSE-214 and CSE-119 cells in duplicate 25 cm^2 flasks. After adsorption for 1 h, unattached virus was removed with three rinses of 2 ml MEM-0. Six ml of fresh MEM-5 was added to each flask. At selected intervals, 0.1 ml was removed from each flask, diluted tenfold in MEM-0, centrifuged at 2000 x g for 10 min, and frozen at -70°C. At the end of the experiment, samples were simultaneously titered by $TCID_{50}$ assay in CHSE-214 cells. An increased titer of virus was detectable at one day after infection in both flasks of CHSE-214 cells (Figure 2). Release of virus from infected cells was shown at two or four days in CSE-119 cells. In contrast to the increase of virus titer of nearly 10^5 in both CHSE-214 flasks, the titer in the flasks of CSE-119 cells increased by less than 10^2. The titer of virus in coho salmon cells decreased from the peak titer by 12 days. Furthermore, the CPE in the CSE-119 cells did not reach completion by 12 days. The virus produced in CSE-119 cells was neutralized by anti-IHNV antiserum. The TR isolate appeared to be capable of limited replication in CSE-119 cells.

Figure 2. The concentrations of infectious virus detected in the supernatant fluids of CHSE-214 (●–●) and CSE-119 (○–○) cell lines after infection with Trinity River infectious hematopoietic necrosis virus. Each curve represents the titers determined in one of a replicate pair of cell cultures.

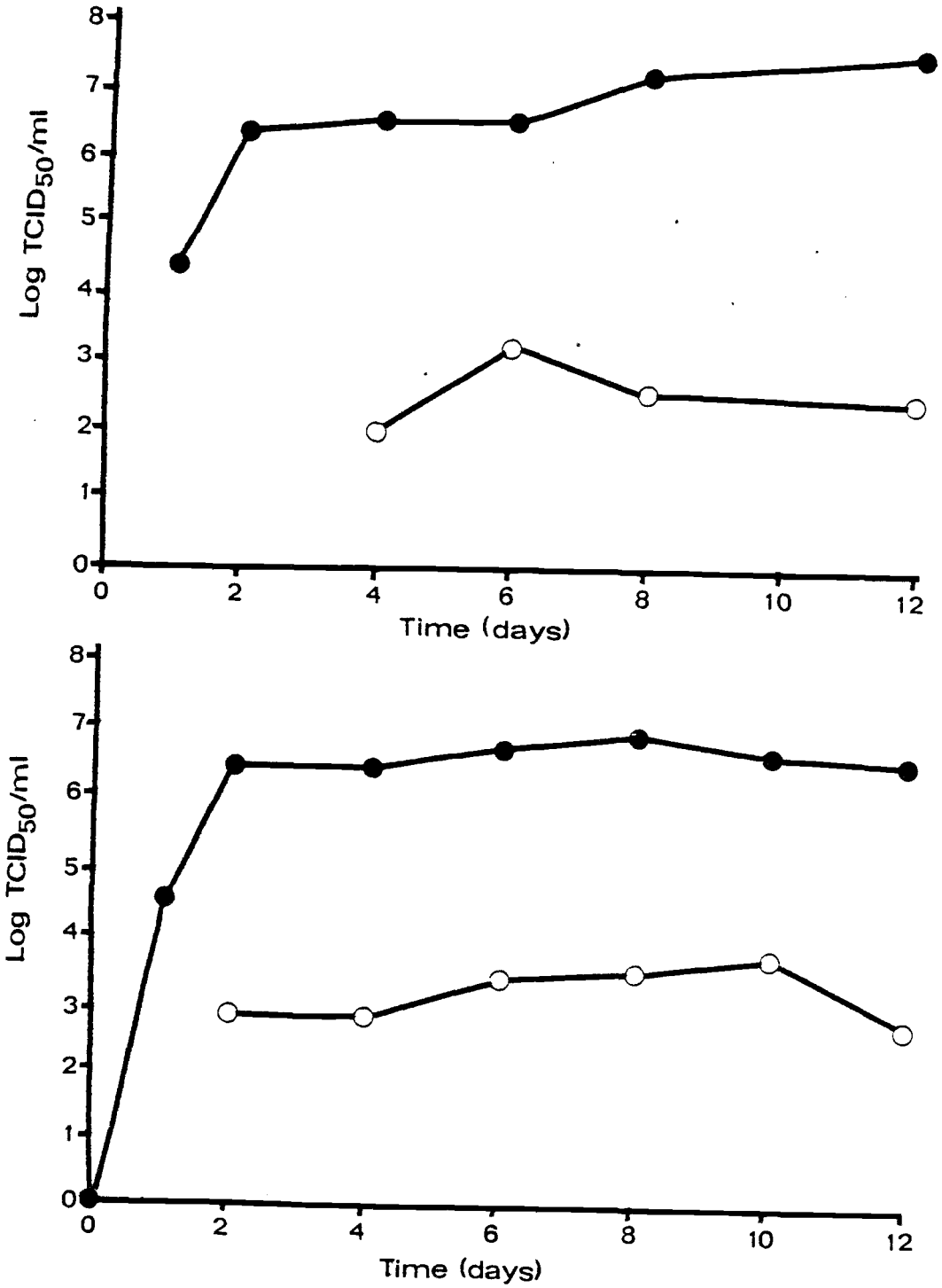

Figure 2.

Plaque Size of Virus

Size of Plaques From Low Passage Virus Isolates

The plaque size of four twice-passed virus isolates was compared in order to determine if virulence could be related to plaque size (Figure 3). The ER isolate was a mixture of large plaques and small plaques. The AK , RB and TR isolates contained predominantly small plaque forms. The approximately bimodal size distribution of ER in a histogram (Figure 4) suggested that the two plaque sizes were distinct types. An arbitrary criterion of 1.55 mm was used to distinguish between large plaques and small plaques in this experiment. The proportions of large plaque virus in the four isolates were: ER;35.5%, RBA;0%, AK;3.3%, TR;0.9%. A chi-square test for the differences in the proportion of large plaque virus showed highly significant differences ($P>.995$, $df=1$).

Purification of Large and Small Plaque Virus

To determine if plaque size was genetically determined, an attempt was made to clone, or purify the two plaque types. Stock large plaque (LP) and small plaque (SP) virus (Figure 5) were grown in CHSE-214 cells after four plaque purification cycles. The size distribution of each virus stock is shown in Figure 6. The LP stock was characterized as being 89% large plaques

Figure 3. Agarose plaque assay of twice-passed Alaska, Round Butte, Trinity River and Elk River infectious hematopoietic necrosis virus in CHSE-214 cells.

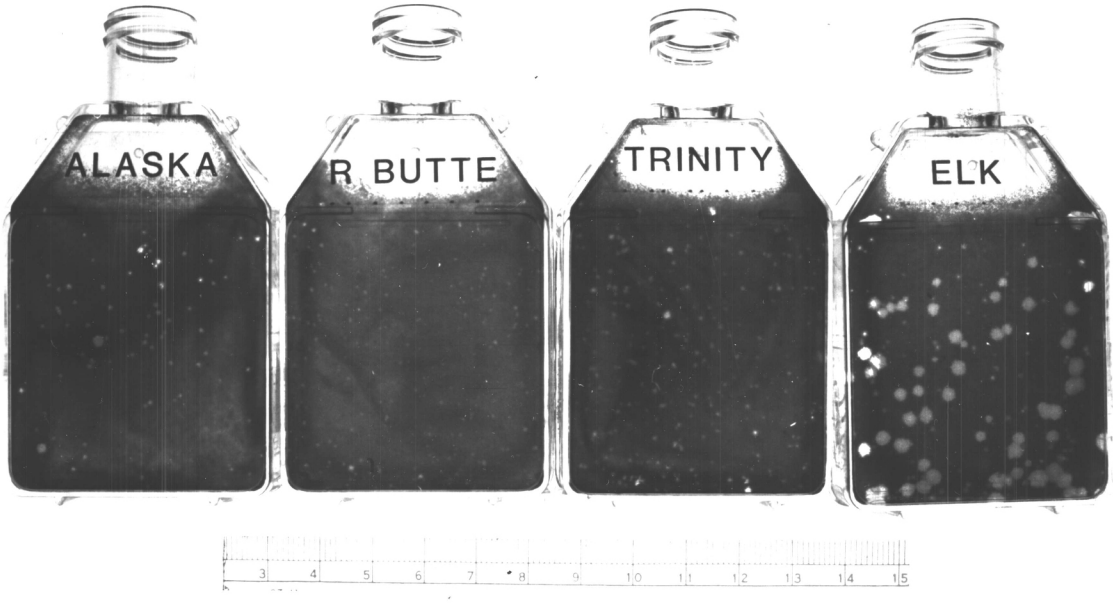

Figure 3.

Figure 4. Distribution of plaque size of Alaska, Round Butte, Trinity River, and Elk River infectious hematopoietic necrosis virus in CHSE-214 cells. The division between large and small plaques is marked by an arrow and equalled 1.55 mm.

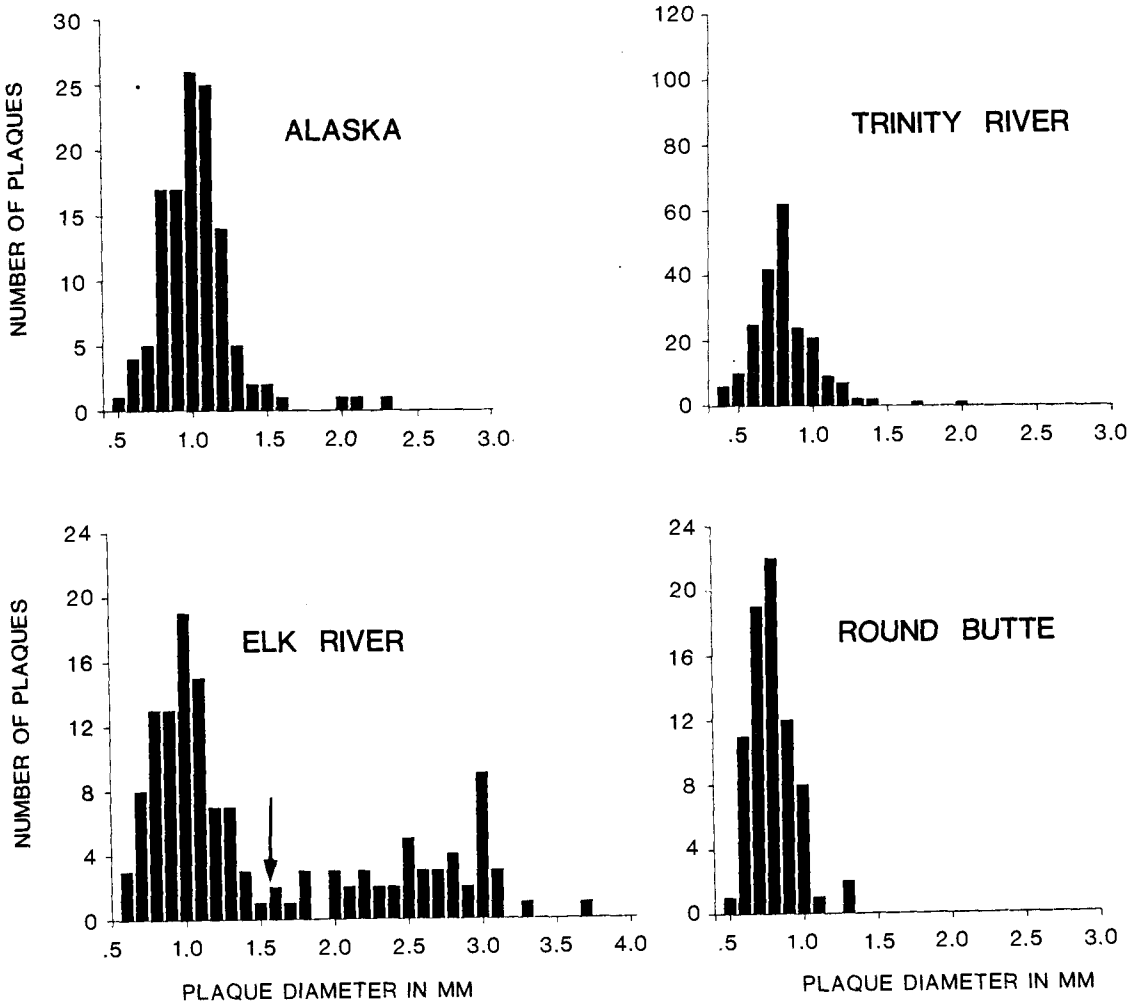
ALASKA
TRINITY RIVER
ELK RIVER
ROUND BUTTE
NUMBER OF PLAQUES
NUMBER OF PLAQUES
PLAQUE DIAMETER IN MM
PLAQUE DIAMETER IN MM

Figure 4.

Figure 5. Agarose plaque assay of large plaque (LP) and small plaque (SP) infectious hematopoietic necrosis virus stocks grown in CHSE-214 cells.

Figure 5.

Figure 6. Distribution of plaque size of large plaque (LP) and small plaque (SP) infectious hematopoietic necrosis virus stocks. The division between large and small plaques is marked by an arrow.

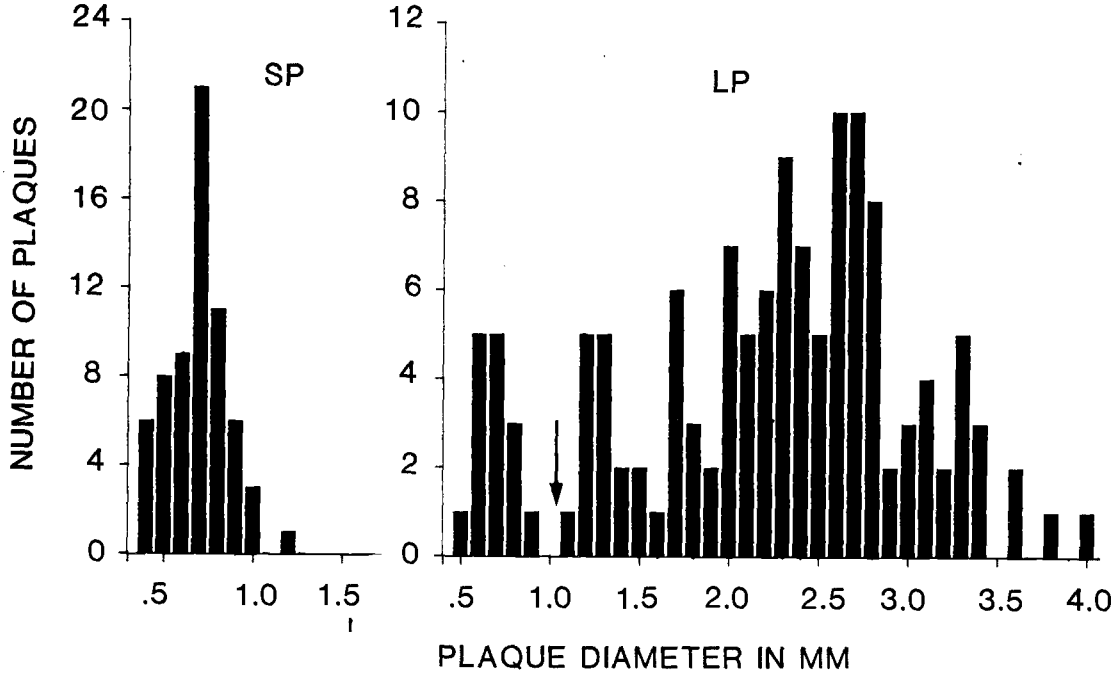
NUMBER OF PLAQUES
SP
LP
24
20
16
12
8
4
0
12
10
8
6
4
2
0
.5
1.0
1.5
.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
PLAQUE DIAMETER IN MM

Figure 6.

by dividing the population at 1.05 mm. This division point was selected because that was where the continuous distribution of sizes in SP ended, and a natural break in the LP population was found. The SP stock contained 1.5% large plaques. In a second experiment using the same virus stocks, the two sizes were divided at 1.55 mm, and the LP and SP stocks were found to contain 82 and 10.4% large plaques respectively.

The differences between the proportions of large plaques in the LP and SP stocks were significant by Chi-square test ($P>.995$, $df=1$). The titer of both stocks was $10^{7.4}$ $TCID_{50}$/ml. No evidence of viral interference such as incomplete CPE was observed in the wells of the $TCID_{50}$ assays. Both LP and SP viruses were neutralized by anti-IHNV antiserum. The effect of cloning on the composition of each population suggested that plaque size was genetically determined, because the plaque size of the progeny virus resembled the parental plaque type.

Increase in Plaque Size With Time

The increase in plaque diameter of the LP and SP stocks was measured over a 12 day period. Twenty flasks per plaque type were inoculated with approximately 20 $TCID_{50}$ each. Flasks were incubated at 16°C after receiving agarose overlay medium. Four LP and SP inoculated flasks were fixed and stained at two day intervals.

No plaques were observed before day six. The mean plaque size of the LP virus increased with time, and achieved a mean diameter of 3.3 mm at 12 days. At all times, the LP virus exhibited significantly larger average plaque size than SP virus (t test $P>.95$, df=1), and the difference between the two types increased with time (Figure 7). The SP virus increased only slightly in size, remaining under one mm. The different rate of increase in plaque diameter under identical conditions further indicated that LP and SP viruses were genetically distinct.

Effect of Temperature on Plaquing of Large and Small Plaque Virus

The effect of temperature on plaquing of LP and SP virus was determined at 6, 12, 16 and 22°C. Five flasks of each type were inoculated with approximately 20 $TCID_{50}$ and incubated for 10 days at each temperature. No plaques were observed at 6°C with either type.

The plaque size of LP virus did not appear different at 12 and 16°C (Table 11). The range of plaque size, mean diameter and percent were similar at both temperatures. There was a significant increase (t test $P>.995$, df=1) in the average number of plaques per flask at 12°C. At 22°C, the average number of plaques was significantly reduced (t test $P>.995$, df=1) compared to 16°C, and only small plaques were observed.

Figure 7. Mean plaque size of large plaque (LP) and small plaque (SP) infectious hematopoietic necrosis virus after selected incubation periods at 16°C. Vertical bars represent the 95% confidence intervals of the means.

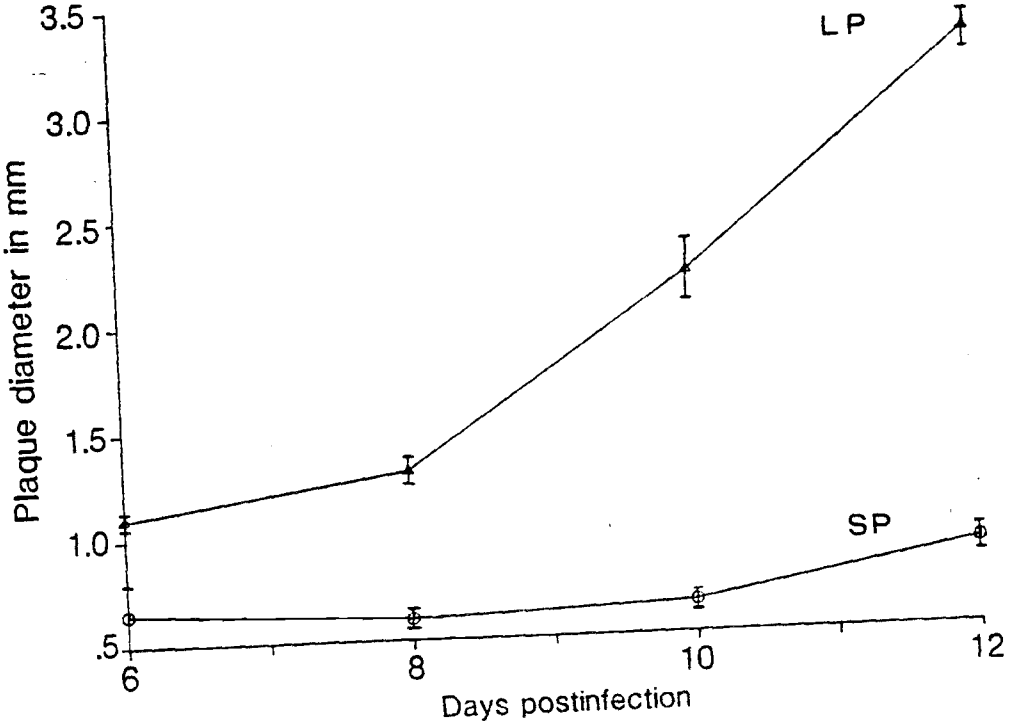
3.5
3.0
2.5
2.0
1.5
1.0
.5
Plaque diameter in mm
LP
SP
6
8
10
12
Days postinfection

Figure 7.

Table 11. Effect of temperature on plaque diameter and number of plaques of large plaque (LP) and small plaque (SP) infectious hematopoietic necrosis virus.

Virus	°C	Range(mm)	Mean Diameter(mm)	SD of Mean[a]	Percent LP	PFU/flask[c]
LP Virus	12	0.4-3.7	2.2	0.06	77	36.4
	16	0.5-3.7	2.4	0.09	83	16.6
	22	0.5-1.3	0.9	0.05	0	3.2
SP Virus	12	0.7-3.0	1.8	0.09	70	12.6
	16	0.6-1.9	1.2	0.04	12	13.4
	22	-	-	-	-	0

[a]Standard deviation of the mean diameter.

[b]Large plaque defined as $\geq$1.6 mm in diameter.

[c]Average of five flasks.

The SP virus exhibited large plaque size when assayed at 12°C (Table 11). The range of plaque size and percent large plaques were similar to the values obtained with LP virus at 12°C. The mean plaque diameter of SP virus was only slightly lower than the mean plaque diameter of LP virus at 12° C, but this difference was statistically significant (t test $P>.95$, $df=1$). At 16°C the SP virus exhibited a characteristic range, mean plaque diameter and percent large plaques. The average number of plaques per flask was similar at 12 and 16°C, but no plaques were observed at 22°C. Phillipon-Fried (1980) reported that CHSE-214 cells grow exponentially at 12, 16 and 23°C, but at 8°C no growth occurs. Thus the effects on plaque size and number at 16 and 22°C were not due to lack of active cell metabolism at those temperatures.

In summary, 6 and 22°C were restrictive for plaque formation of either LP or SP virus, but a small proportion of LP virus formed small plaques at 22°C. The plaque size of LP virus was large at 12 and 16°C, but efficiency of plaquing under agarose overlays was greater at 12°C. The SP virus was similar in plaque size to the LP virus at 12°C. The efficiency of plaquing of SP virus at 12 and 16°C was the same. The SP virus appeared to be more temperature sensitive than the LP virus.

Definition of Plaque Size Phenotype as Wild Type or Mutant

The purification of SP and LP forms of IHNV from the same isolate suggested that one form might be a mutant of the other form. Evidence was sought to determine which plaque type was the wild type or the numerically dominant type in infected fish. Numerical dominance of small plaque virus was observed in four low-passage IHNV strains (Figure 3). Large plaques were numerically prominent in ER, which was the only one of the four isolates derived from an outbreak of IHNV in juvenile fish. Two experiments were carried out to determine if the large virus was associated with mortality of juvenile fish.

Unpassed viruses from steelhead trout ovarian fluid (RBA) and naturally infected dead juvenile steelheads (RBJ) were plaqued under agarose overlay at 16^{o}C (Figure 8). The plaque size range of RBA was 0.5 mm to 1.6 mm, and RBJ plaques varied from 0.5 mm to 1.6 mm in diameter (Figure 9). Clearly, large plaques were not associated with virus from adult or juvenile steelhead trout in this experiment.

Unpassed virus from experimentally induced juvenile chinook salmon mortalities was compared to the twice-passed ER virus used to infect the fish. The waterborne exposure was carried out at 12^{o}C, a temperature permissive for growth of both large and small plaque virus in the temperature experiments. While the cell culture grown ER

Figure 8. Agarose plaque assay of unpassed Round Butte infectious hematopoietic necrosis virus from adult and juvenile steelhead trout.

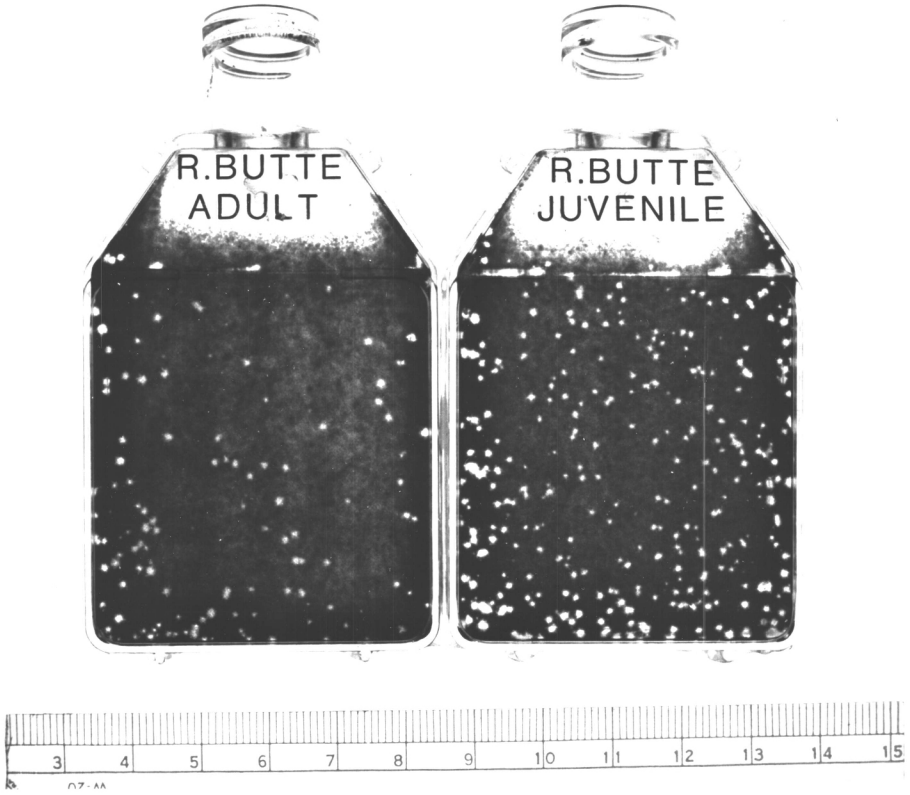

Figure 8.

Figure 9. Distribution of plaque size produced by unpassed Round Butte infectious hematopoietic necrosis virus from adult (A) and juvenile steelhead trout (B).

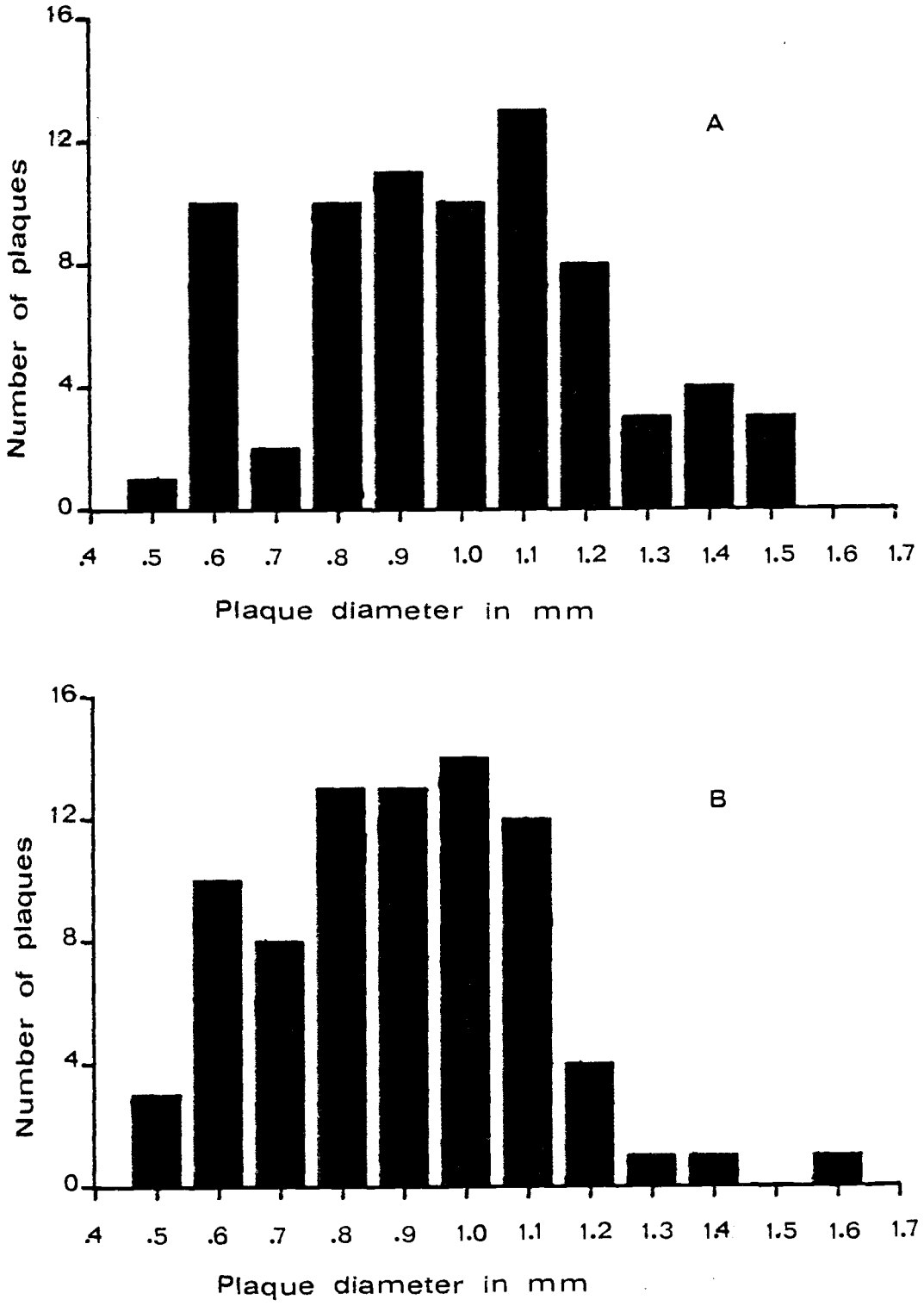
16
12
8
4
0
Number of plaques
A
.4 .5 .6 .7 .8 .9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7
Plaque diameter in mm
16
12
8
4
0
Number of plaques
B
.4 .5 .6 .7 .8 .9 1.0 1.1 1.2 1.3 1.4 1.5 1.6 1.7
Plaque diameter in mm

Figure 9.

was a mixture of large plaque (23%) and small plaque virus, very few large plaques (1.9%) were present in virus recovered from dead fish (Figure 10). The size division between SP and LP (1.9 mm) was based on the fact that the continuous distribution of SP ended at that size, and there was a natural break in the LP population at that diameter. The shift in favor of the SP from was highly significant (Chi-square test>.995, df=1). It was not known whether this rapid shift was due to selection or mutation. Small plaques were the predominant form found in dead chinook salmon (Figure 11). These two experiments, involving IHNV from two different watersheds, and two different host species, strongly suggested that the small plaque-forming virus is the wild type and that large plaques represent a mutant.

Effect of pH on Plaque Size

Because the plaque size of some viruses is affected by pH, experiments were conducted to determine if the plaque size was determined by the pH of the agar overlay. The comparison of ER from cell culture and after infection showed that cell culture grown ER was a mixture of large and small plaque virus, but ER from dead fish was almost all small plaque virus. Replicate flasks from the same experiment were incubated with the caps of the flasks loosely fastened, allowing the CO_2 concentration to

Figure 10. Distribution of plaque size of Elk River infectious hematopoietic necrosis virus from chinook salmon (A) exposed to virus passed twice in CHSE-214 cells (B). The division between large and small plaques is marked by an arrow.

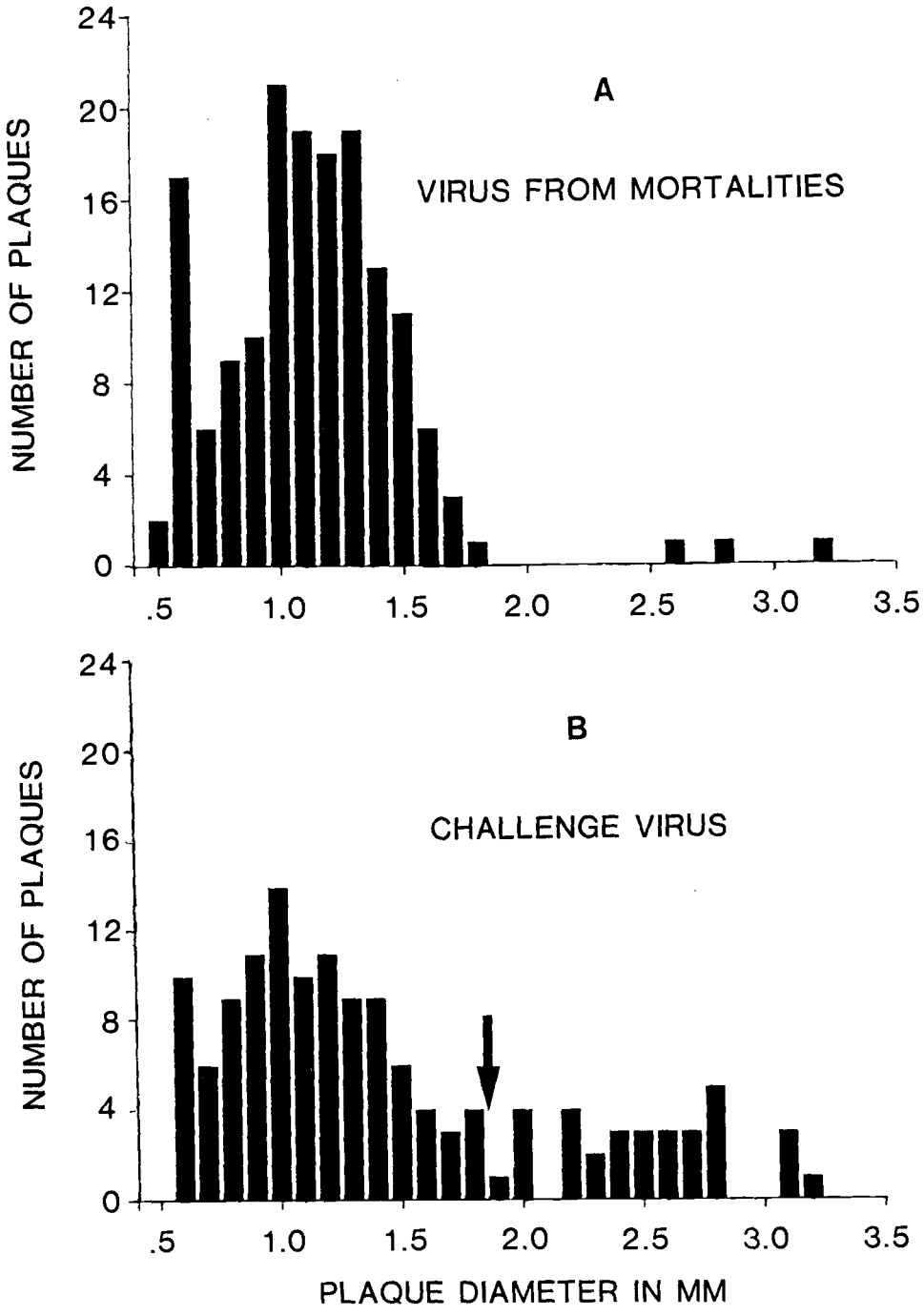
A
VIRUS FROM MORTALITIES
NUMBER OF PLAQUES
24
20
16
12
8
4
0
.5
1.0
1.5
2.0
2.5
3.0
3.5
B
CHALLENGE VIRUS
NUMBER OF PLAQUES
PLAQUE DIAMETER IN MM

Figure 10.

Figure 11. Agarose plaque assay of Elk River strain of infectious hematopoietic necrosis virus after two passages (P2) in CHSE-214 cells, and from chinook salmon mortalities (MORT) after exposure to twice-passed virus. The inner two flasks were incubated with tightly-sealed caps, the outer flasks with caps on loosely.

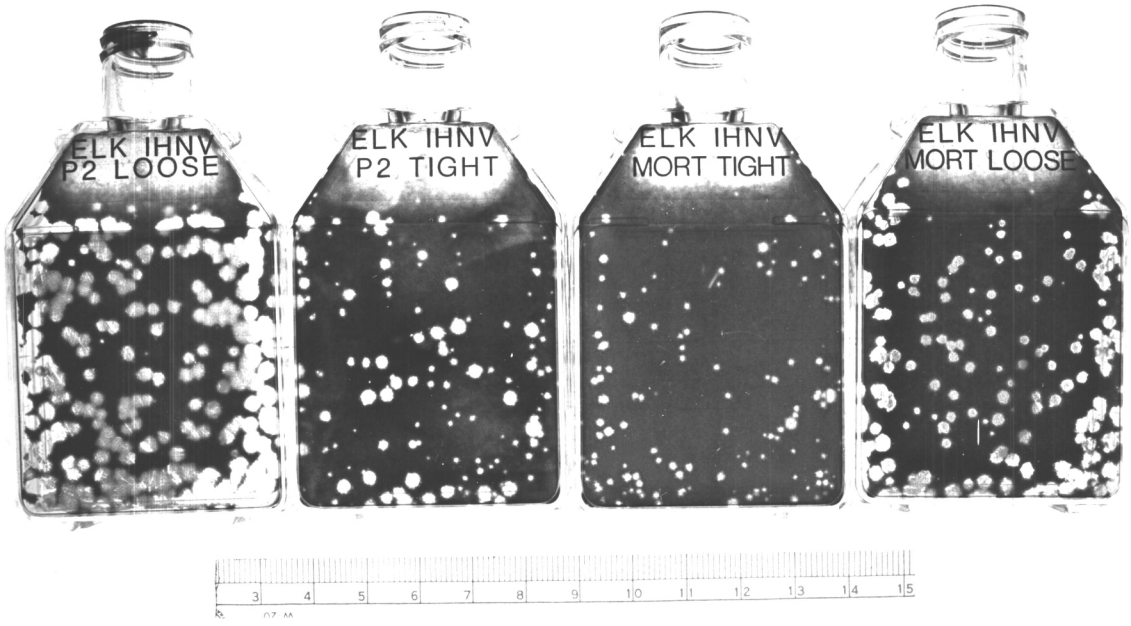
ELK IHNV
P2 LOOSE
ELK IHNV
P2 TIGHT
ELK IHNV
MORT TIGHT
ELK IHNV
MORT LOOSE

Figure 11.

equilibrate with the outside atmostphere. According to visual observation of the phenol red indicator, the overlay in the loosely capped flasks remained alkaline over the 10 day period, compared to the tightly capped flasks, which became progressively more acidic. The plaques in the loosely capped flasks were visibly larger than the plaques in the tightly capped flasks (Figure 11). The small plaque virus from dead fish displayed large plaque morphology in the loosely capped flasks. Thus, the effect of incubation at 12 and 16^{o}C on plaque size was reproduced at 16^{o}C by varying the pH of the overlay.

The temperature effect on plaque size could actually reflect pH sensitivity. The metabolic rate of the CHSE-214 cells might be expected to rise with increasing temperature of incubation, with a resultant increase in the generation of acidic byproducts or CO_2. To test the relationship between temperature of incubation and acidification of the medium, a mock plaque assay was performed in CHSE-214 cells, with 8 ml of MEM-5 replacing the MEM-5 agarose overlay. Flasks were incubated at 12, 16 and 20^{o}C. After 3, 7 and 10 days of incubation, the pH of duplicate flasks was measured for each temperature. Figure 12 shows that the average pH of the medium declined with increasing time of incubation at all three temperatures, but at all times, the pH was lower in flasks incubated at higher temperatures. The final pH of the 12,

Figure 12. Decline in the pH of bicarbonate buffered MEM-5 medium after incubation at 12 (●–●), 16 (○–○) and 20 (▲–▲) ^{o}C. Each point represents the average of two flasks of CHSE-214 cells.

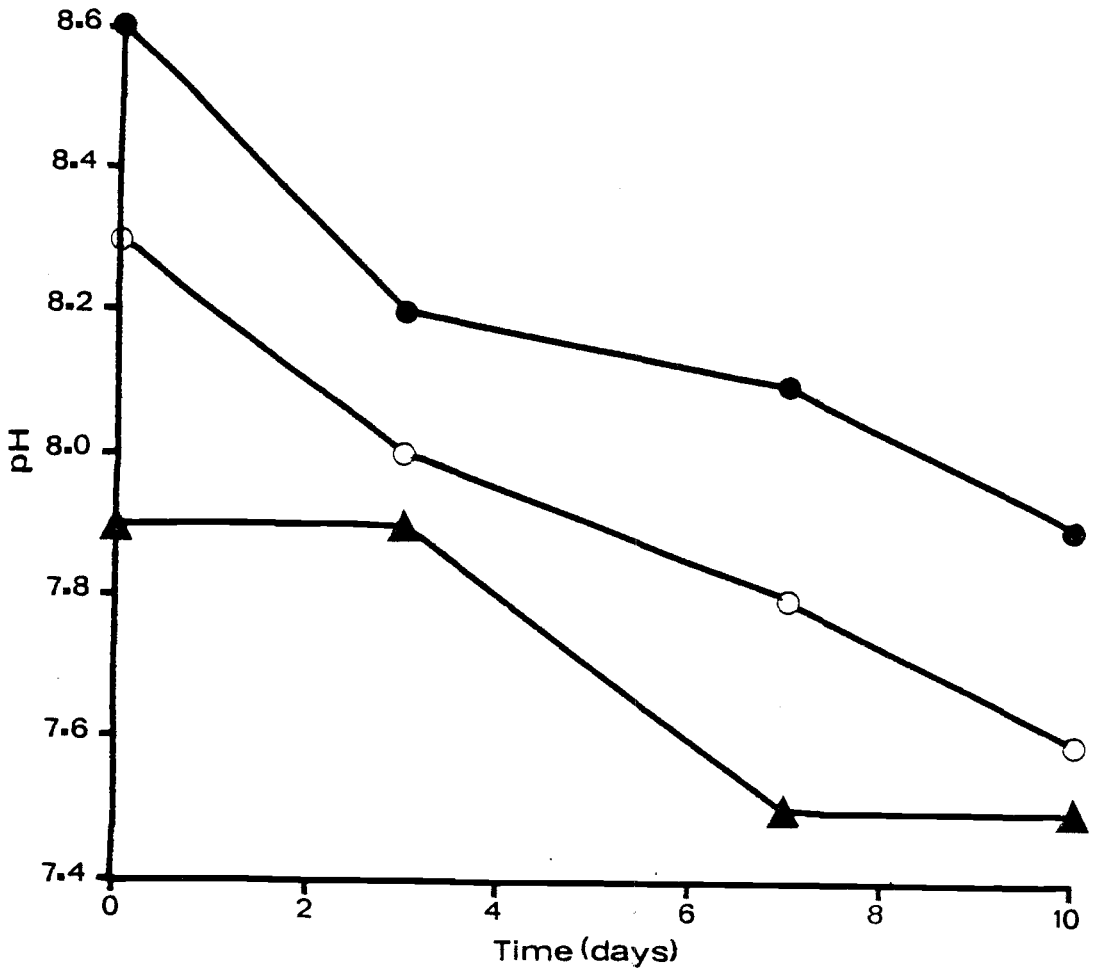

Figure 12.

16 and 20°C flasks was 7.9, 7.65 and 7.45 respectively. In view of the effects of incubation temperature on plaque size, it was suspected that a pH of 7.9 or higher would allow the SP virus to exhibit LP morphology, while a pH of 7.65 or lower would allow the SP and LP viruses to be distinguished by size.

A means of varying the pH of the overlay at a single temperature was desired in order to test the effect of pH on plaque size. Agarose overlay medium was prepared with 0.028 M HEPES buffer (Sigma Chemical Co.) in addition to the usual 0.038% $NaHCO_3$. Replicate flasks infected with approximately 20 $TCID_{50}$ of LP and SP viruses were overlaid with MEM-5-HEPES with agarose at pH 7.5 and 8.3. In order to maintain a pH of 8.3 without a CO_2 incubator, these flasks were loosely capped (Wolf and Quimby, 1973). A mock plaque assay using the MEM-5-HEPES without agarose confirmed that over the 10 day incubation period, the pH of the medium remained close to the desired levels (Figure 13).

In this experiment, the division between large and small plaques was defined at $\geq$0.8 mm by using a histogram of the LP virus at pH 7.5. This division was made in order to match the lowest previously measured proportion of large plaque virus in the LP stock (83%). The LP virus stock produced predominantly large plaques at both pH 7.5 and 8.3 (Figure 14, Table 12). The mean plaque diameter

Figure 13. The pH of HEPES-bicarbonate buffered MEM-5 medium after incubation at 16°C. Each point represents one flask of CHSE-214 cells. The flasks initiated at pH 7.5 (●–●) were incubated with tightly sealed caps. The flasks initiated at pH 8.3 (○–○) were incubated with caps on loosely.

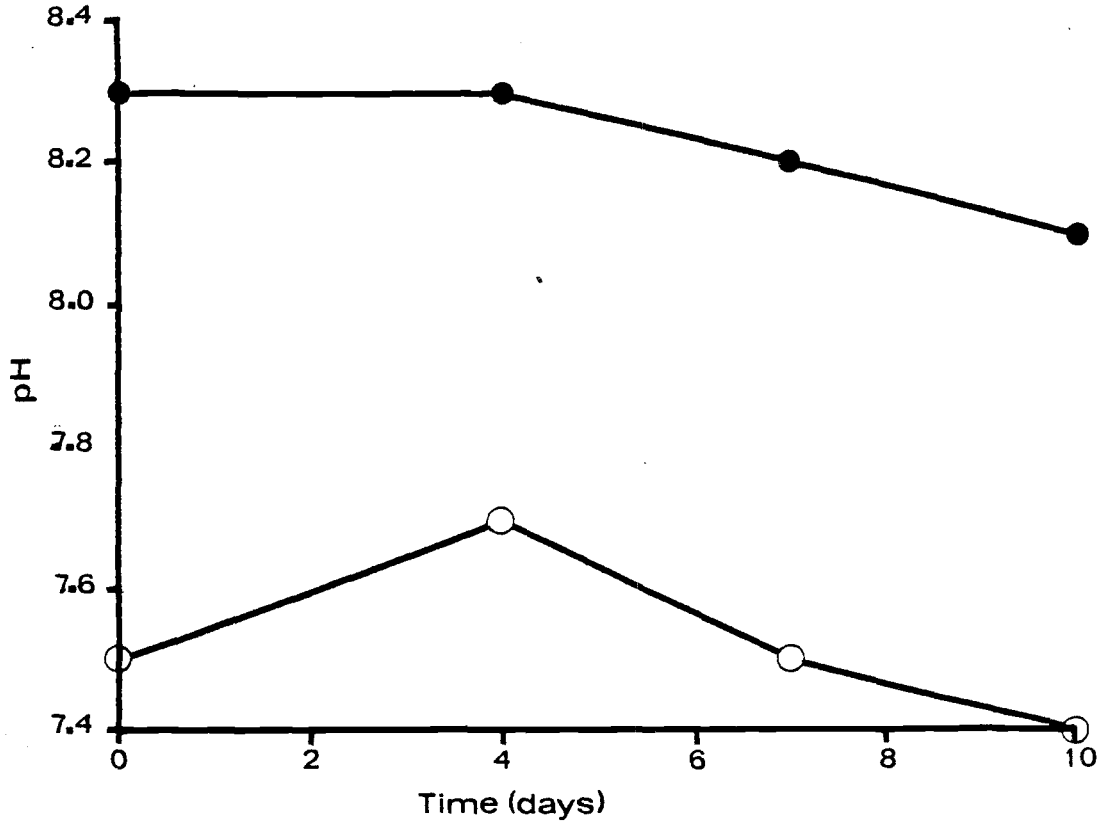

Figure 13.

Figure 14. Agarose plaque assay of large plaque (LP) and small plaque (SP) infectious hematopoietic necrosis virus. The monolayers of CHSE-214 cells were overlaid with HEPES-bicarbonate buffered medium adjusted to the indicated pH and incubated at 16°C. The flasks initiated at pH 8.3 were incubated with caps on loosely.

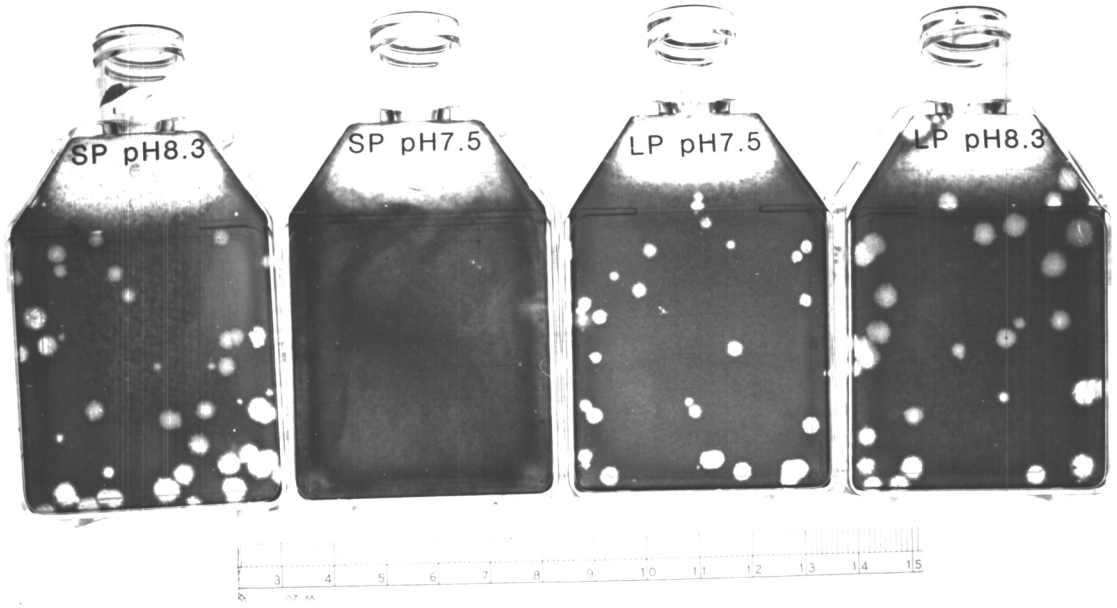

Figure 14.

Table 12. Effect of overlay pH on plaque diameter and number of plaques of large plaque (LP) and small plaque (SP) infectious hematopoietic necrosis virus.

Virus	pH	Range(mm)	Mean Diameter(mm)	SD of Mean[a]	Percent LP	PFU/Flask[b]
LP Virus	7.5	0.4-4.6	2.16	0.13	79	16.7
	8.3	0.5-5.7	3.98	0.13	93	25.7
SP Virus	7.5	0.4-0.8	0.55	0.21	0	5.7
	8.3	0.7-5.5	3.03	0.16	92	17.3

[a]Standard deviation of the mean.

[b]Large plaque defined as $\geq$0.9 mm in diameter.

[c]Average of three flasks.

of the LP virus was significantly greater at the more alkaline pH (t test, $t > t_{.995}$, df=179). The SP virus stock also had a greater mean plaque diameter (t test, $t > t_{.995}$, df=88) at pH 8.3. Further, both LP and SP viruses produced more plaques at the more alkaline pH. Most importantly, the percent large plaques was about equal in both LP and SP viruses at pH 8.3, but at pH 7.5, no large plaques were observed in the SP virus plaque assay. Therefore, the wild type SP virus appeared to be more pH dependent with respect to plaque diameter than the mutant LP virus. The smaller mean plaque diameter of LP virus at pH 7.5 was probably due to the portion (21%) of the population composed of small plaque-forming virus which forms large plaques at pH 8.3. However, the larger overall plaque size range of LP virus at pH 8.3 suggested that some virus which forms large plaques at pH 7.5 forms even larger plaques at pH 8.3. A similar proportion (7-8%) of virus in both LP and SP virus stocks did not respond to the more alkaline condition by producing large plaques. This suggested that a third plaque type may exist which forms small plaques at either pH.

DISCUSSION

Dose-dependent mortality was observed when juvenile salmonid fish were exposed to TR. This is in contrast to the results of Amend and Nelson (1977) who found that 70-100% of sockeye salmon exposed to virus concentrations as low as $10^{2.8}$ $TCID_{50}$/ml died. In the present study, a wider range of virus concentration was employed (10^{5}-10^{1} $TCID_{50}$/ml). The demonstration of dose-dependent mortality probably resulted from exposure of fish to concentrations of virus both above and below the LD_{50}, which was found to be as low as 10^{2} $TCID_{50}$/ml in sockeye (kokanee) salmon (Table 3).

The relative lack of susceptibility of all three chinook salmon stocks tested to AK and RBA probably explains the lack of recorded viral outbreaks in chinook salmon in Alaska and at Round Butte hatchery. Virus associated with naturally occurring sockeye salmon epizootics has been shown to possess little virulence for experimentally exposed chinook salmon (Rucker et al., 1953; Wingfield et al., 1970; Wertheimer and Winton, 1982). The Alaska and Round Butte viruses are probably representative of a group of IHNV isolates which are virulent for anadromous and non-migratory sockeye salmon and also steelhead trout, but are comparatively avirulent in chinook salmon.

The three IHNV isolates from sites of chinook salmon mortality, ER, TR, and FE, caused deaths in all salmonid species and fish stocks tested, with the possible exception of coho salmon. Comparable results were obtained by Wingfield and Chan (1970), who demonstrated experimentally that the SRCD (IHNV) virus killed chinook salmon, sockeye salmon, and steelhead trout, not coho salmon. The results of the present and previous investigations suggest that there exists a group of IHNV isolates which are virulent for chinook salmon, as well as sockeye salmon and steelhead trout.

Elk River and Round Butte chinook salmon exhibited a similar pattern of susceptibility to the different virus isolates. Therefore, the lack of chinook salmon mortality at Round Butte and the historical mortality at Elk River does not indicate that Round Butte chinook salmon are resistant to all isolates of IHNV. The difference in the history of the two stocks can be explained by the different host specificity of RBA and ER.

Strains of IHNV with differing host specificities might act as different agents if present in the same watershed. For this reason transfer of fish or diversion of water even between two areas where IHNV is present should be regarded with caution. Because determinations of virulence can be greatly affected by the species tested, IHNV strains which are being considered for use as

attenuated vaccines should be evaluated in different salmonid species. For example, AK was shown to act as an attenuated virus in chinook salmon, and protection was demonstrated. However, AK was shown experimentally to be virulent in steelhead trout and sockeye salmon, and these species are often present in the same waters as chinook salmon.

Mortality associated with IHNV has not been previously observed in coho salmon following injection (Watson et al., 1954; Parisot and Pelnar, 1962) or waterborne exposure (Wingfield et al., 1970; Wingfield and Chan, 1970). The failure to isolate IHNV from approximately half of the dead fish and the observation of concurrent fungus infections prevented a definite conclusion that IHNV is by itself capable of killing juvenile coho salmon. However, virus was isolated from dead and asymptomatic fish following waterborne exposure to high concentrations of IHNV, indicating that infection did occur.

Some of the typical external signs of IHNV infection were reduced or absent in coho salmon. The histopathology was also not typical of disease caused by IHNV in other salmonid species. However, Yasutake (1978) reported morbidity and mortality in both naturally and experimentally infected sockeye salmon accompanied by a lack of external signs and only minor tissue changes. The sockeye salmon

studied were 7-14 months old. Salmonid fish of this age are usually not affected by IHNV, but such uncharacteristic infections are increasing in frequency and have histopathological and clinical manifestations unlike those associated with epizootics in younger fish (Yasutake, 1978). Perhaps coho salmon attain their relative resistance to IHNV at a much earlier age than other salmonid fish, as indicated by the lack of mortality observed in this study when 0.45 gm and larger fish were exposed to IHNV. The atypical course of the disease may reflect this resistance.

Studies examining the adaptation of virus to a new host have initially found minimal signs of infection but lethal infections can be produced after serial passage (Fenner et al., 1974). For example, after the experimental transmission of poliomyelitis virus from a primate to a cotton rat, Armstrong (1939) was able to observe signs in only one of 11 rats inoculated with infective rat material; the virus was lost on subsequent passage in rats. Later experiments showed that successive transfers led to increased virulence for the rat. Certain features of infection of an atypical host were also apparent in the experiments with coho salmon. The small number of fish that died, the reduced clinical signs and histopathology, and the 50% reisolation rate indicated that coho salmon are a resistant host for IHNV at the present time.

The apparent genetic transfer of virus resistance from coho salmon to the rainbow trout egg by way of coho salmon sperm reinforces the concept that a host species factor(s) can determine resistance to IHNV. This observation further demonstrates that resistance to rhabdoviruses infecting fish can be genetically transferred by sperm (McIntyre and Amend, 1978; Ord, et al., 1976). Further studies to identify the resistance factor could make possible the cloning of the gene involved and the transfer of IHNV resistance to the more susceptible salmonid species without the disadvantages of hybridization, which include low hatching success, congenital malformations and sterility (Buss and Wright, 1956). The hatching success of the hybrids (2%) was much lower than the 54% reported by Blanc and Chevassus (1979). However, in more recent experiments, the same group of researchers have found a 0% hatching rate for the coho-salmon-rainbow trout hybrid (Chevassus, et al., 1983). Variability in fertilization success, hatching and length of development of coho salmon-rainbow trout hybrids was shown to be of maternal origin (Chevassus and Petit, 1975). The use of a single rainbow trout female as the sole source of eggs may have contributed to the low hatching rate. Satisfactory hatching of coho salmon and rainbow trout from the same pooled coho salmon sperm and rainbow trout eggs used in hybridization showed that the gametes used were viable.

Brownstein (1983) crossbred a strain of mice resistant to respiratory infection with Sendai virus with a susceptible mouse strain. After intranasal infection, the F_1 hybrids and the resistant parental strain showed equal survival. Further experiments indicated that resistance behaved as a single dominant autosomal trait which mapped in chromosome one, and was not linked to the major histocompatability complex H-2. The F_1 coho salmon-rainbow trout hybrids were as resistant as the coho salmon, and may be similar to the system described by Brownstein (1983). Induced triploidy increases the survival of interspecific fish hybrids (Scheerer and Thorgaard, 1983) and may increase the number of F_1 hybrids available for study. However, potential F_1 sterility and the long generation time of salmonid fish are limitations to further experiments.

The titer of the virus isolates in CHSE-214, SSE-5 and STE-137 cells suggested that AK and RBA had a reduced ability to replicate in CHSE-214 cells, compared to ER, FE and TR. This was especially apparent in the efficiency of plaquing experiments (Table 9, 10). Nims et al., (1970) also found that a chinook salmon embryo cell line (CHSE-114) produced 10^{-2}-10^{-3} as much virus as SSE-5 or STE-137 cells after infection by an IHNV isolate from sockeye salmon. The reduced sensitivity of CHSE-214 cells to AK and RBA is in agreement with the avirulence of the two

isolates for juvenile chinook salmon. However, asymptomatically infected adult chinook salmon have been found at the sites where AK and RBA were isolated (Mulcahy et al., 1980; Wertheimer and Winton, 1982).

The efficiency of plaquing data indicated that CHSE-214 cells were more sensitive to ER and FE than STE-137 cells. The TR isolate was similar to ER and FE in its ability to form plaques in CHSE-214 cells. The host specificities of all five isolates were not as obvious when $TCID_{50}$ assays were used, perhaps because the prolonged exposure of cells to virus in this procedure tended to overcome the factors responsible for specificity. However, Fendrick et al., (1982) reported that ER plated directly from naturally infected chinook salmon had a higher titer in CHSE-214 cells than STE-137 by $TCID_{50}$ assay.

Possible host specificities of IHNV should be considered when salmonid cell lines are used in certification of fish stocks as free of IHNV infection. In areas where virus is endemic in wild sockeye salmon or areas where mortalities recur in rainbow and steelhead trout, the use of CHSE-214 cells may result in a reduction in the chances of IHNV detection. In both $TCIO_{50}$ and plaque assays, the SSE-5 cell line was highly sensitive to all five isolates studied. This sensitivity may reflect the susceptibility of sockeye salmon to different IHNV

isolates as shown in the present study and reported by others (Wingfield et al., 1970; Wingfield and Chan, 1970).

Wingfield et al. (1970) concluded that no increase in IHNV titer was detected in CSE-119 cells exposed to the Oregon sockeye IHNV. In the present study, increases in titer of approximately one $\log_{10}$ were observed after CSE-119 cells were infected with TR and AK. This increase in titer contrasted to the seven $\log_{10}$ increase found in similarly infected CHSE-214 cells. Possible reasons for the difference in results may be that different isolates were used in the previous report, and that in the present study, cells were infected two days after trypsinization, rather than at four to seven days after trypsinization. No IHNV plaques were observed by Phillipon-Fried (1980) in CSE-119 cells, and a similar result was found in this study when four virus isolates were tested in CSE-119 cells. The limited replication and incomplete CPE seen in infected CSE-119 cells may help to explain the lack of external disease signs and 50% incidence of virus re-isolation in coho salmon experimentally exposed to IHNV. The relative resistance of CSE-119 cells suggests that the resistance of coho salmon to IHNV is at least partially determined at the cellular level. Some similarities exist between the resistance of CSE-119 cells and the resistance of a monkey cell line to host restricted enteroviruses (Hsiung and Melnick, 1957a). No plaques can be observed

in either case, but replication of the restricted virus occurs after a high virus inoculum with very limited CPE.

The host-specific and virus-specific factors suggested by the virulence determinations appear to operate at the cellular level. This implies that resistance of chinook salmon to RBA and AK cannot be completely ascribed to the immune response. The nature of the factors resulting in host specificity of IHNV was not determined in this study. However, the asymptomatic infections of chinook salmon adults and the ability of RBA and AK to replicate and form plaques in CHSE-214 suggests that the mechanism of resistance is not solely due to lack of compatible cellular receptors and viral envelope proteins.

The cell line specificity of infectious pancreatic necrosis virus (IPNV), which is a serious pathogen of salmonid fish, was studied by Scheerer and Cohen (1975). Less than 0.01% of virus grown in rainbow trout gonad cells (RTG-2) was able to form plaques in a cell line from Pimephales promelas, the fathead minnow (FHM). Once adapted to FHM cells, however, IPNV formed plaques with equal frequency in both cell lines. No difference could be detected in morphology, buoyant density and reactivity with specific antiserum of RTG-2 and FHM-adapted IPNV. Most of the difference in the efficiency of plaquing was due to a failure of RTG-2-adapted virus to adsorb to FHM cells, but much of the adsorbed virus also failed to form

plaques. Virus capable of infecting FHM cells was produced by high frequency (10^{-4}-10^{-5}) mutations in the RTG-2-adapted virus, which were rapidly selected for in FHM cells. The difference in the ability of RBA to form plaques in SSE-5 and CHSE-214 cells were not of the same magnitude as the IPNV system, possibly due to growth of the virus stock in CHSE-214 cells. However, the difference was highly significant. The $TCID_{50}$ titer of TR and AK in CSE-119 cells was less than 0.1% of the titer in SSE-5 cells. The causes of the cell line specificity, and ultimately the host specificity, of IHNV could be similar to those shown in the IPNV system.

The genetic basis associated with the large and small plaques produced by IHNV was demonstrated by cloning of predominantly large or small plaque virus stocks. The residual amounts of unselected virus present in the LP and SP virus stocks may have been due to back mutation, which can occur at frequencies up to 10^{-2} (Fenner et al., 1974), and growth of the revertants in the liquid-phase cell cultures inoculated with agar plugs (Walen, 1963). The different rates of increase in plaque size of the LP and SP virus over time further suggested that the two plaque types were genetically distinct.

A variety of factors in the plaque assay have been shown to result in genetically determined plaque size variation. Interferon can determine the plaque size of

mutants in two ways (Takemoto, 1966). Small plaque mutants may be more efficient interferon inducers, and can be more sensitive to the effect of interferon than the large plaque virus. The CHSE-214 cells used in the plaque size experiments do not produce interferon (MacDonald and Kennedy, 1979). The d or acid-sensitive characteristic of poliovirus reported by Vogt et al. (1957), was later shown to be due to binding of virus at neutral or slightly acidic pH by sulfated polysaccharide components present in agar (Agol and Chumakova 1962). Resistant large plaque forms of poliovirus and members of other virus groups affected by sulfated agar polysaccharide occur spontaneously (Takemoto, 1966). The IHNV plaque size experiments reported here were performed with agarose, which is free of the sulfated polysaccharide inhibitor. A difference in the rate of release from infected cells was reported in large and small plaque mutants of vesicular exanthema of swine virus (McClain and Hackett, 1959). The rate of release of the large plaque form was more rapid at pH 7 and 8, while the small plaque mutant was cell associated at pH 7. The large plaque form was also more stable at the lower pH than the small plaque mutant.

Temperature above or below the optimum growth temperature can cause reductions in plaque size and number (Takemoto, 1966). The SP virus initially appeared to be more sensitive with regard to plaque size than the LP

virus to temperatures of 16°C and above. At 22°C, the SP virus did not form plaques. However,the pH of the medium in flasks of CHSE-214 cells was shown to decline with increasing incubation temperature. The effect of temperature on the plaque size and number of plaques of SP virus were produced at a single temperature by varying the pH of the overlay medium. The pH was varied two ways: by incubating flasks with loose caps, resulting in alkaline conditions, and by initiating the experiment with HEPES-buffered media adjusted to pH 7.5 and 8.3. In both experiments, the size and number of LP virus plaques was less affected at the more neutral pH than those of SP virus. Therefore, the apparent temperature sensitivity of SP may actually be a pH sensitivity. This pH sensitivity appears to be the factor determining the plaque size of both SP and LP virus. Partially open flasks were used to help generate alkaline conditions in the present study. This may have altered other factors as well as pH. Further experiments with incubation in open vessels under partial pressures of CO_2, or using a medium with additional buffering capacity would confirm the pH sensitivity of SP virus. The pH effect was probably due to a change in the ability of the CHSE-214 cells to replicate SP virus rather than a pH lability of extracellular virus, because the survival of extracellular IHNV was found to be unaffected by pH 6-8 (Pietsch et al., 1977).

Experiments indicated that the SP virus was a wild type, virulent form of IHNV. Small plaques were the predominant plaque form observed in four twice-passed isolates. Each of these twice-passed isolates proved to be virulent for the species most affected at the site of isolation. The recovery of small IHNV plaques from a naturally occuring epizootic in juvenile steelhead trout further indicates that the small plaque form is virulent. Also, naturally infected adult steelhead trout shed the small plaque form in the ovarian fluid. Thus, both life stages of the host from which IHNV can usually be isolated were infected with small plaque virus. The proportion of large plaques in ER was significantly reduced after host passage, again suggesting that the small plaque is the virulent, wild type form. Exposing fish to LP and SP virus did not yield conclusive results regarding the relative virulence of the two plaque types, possibly because the residual small plaque virus present in the LP virus may be rapidly selected for in the host at 12^{o}C.

The evidence that the SP virus may represent the virulent, wild type IHNV contrasts to the general case in animal viruses (Takemoto, 1966), including other rhabdovirus systems (Flamand, 1980) in which small plaques are formed by attenuated mutants. Another example of an exception to this general rule was reported by Douglas, et al., (1974). Rhinovirus type 15 passaged twice in cell

culture was composed of 30% large plaques. The proportion of large plaques rose to 60% after three passages, and the third-passage preparation was attenuated in comparison to the second-passage virus stock. Cloning of each plaque type four times resulted in two populations with 2% and 93% large plaque types. The large plaque virus stock was attenuated in comparison to the small plaque virus stock. Analysis of rhinovirus shed by subjects inoculated with the large plaque virus stock showed a predominance of small plaque forming virus, indicating rapid reversion or selection in favor of the wild type. The results of the study of large plaque rhinovirus type 15 are similar to those presented here on the properties of LP IHNV.

The possible effect of the presence of large plaque forms on the comparative virulence results should be considered. The ER contained a higher proportion (35.5%) of large plaque forms than RBA, AK and TR (<5%). The isolate found to be least virulent in sockeye salmon and steelhead trout was ER. Of the three isolates which were virulent in chinook salmon, ER was less virulent than TR and FE. It is possible that the large plaque forming virus in ER reduced the virulence of that isolate for all species tested. However, the infective dose determinations and the efficiency of plaquing results support the conclusion that ER belongs in the group of isolates virulent in chinook salmon, and also in sockeye salmon and in steelhead trout.

Wolf and Quimby (1973) emphasized the importance of control of pH for plaquing of fish viruses. If the wild type, small plaque IHNV in the host is pH sensitive, then isolation of virus for diagnostic or certification purposes should be performed at a pH of 8.0 or above. In the present study, virus directly plated from naturally infected hosts was not tested for pH sensitivity. However, virus directly plated from experimentally infected hosts was pH sensitive, and produced larger plaques at the higher pH (Figure 11). In contrast to the sensitivity of the plaque assay to pH of 7.5 reported here, Burke and Mulcahy (1980) observed a greater number of plaques at pH 6.8 than at pH 7.4, using epithelioma papillosum carpio (EPC) cells. It may be that EPC cells are more sensitive to IHNV than CHSE-214 cells (Fendrick et al., 1982) because a pH near neutrality does not reduce the ability of the cells to support IHNV replication.

Leong et al. (1981a) reported that a IHNV stock was composed of large and small plaque types. The small plaque form did not plaque well under agarose overlay, but was readily detectable when CHSE-214 cells pretreated with a polycation were overlaid with tragacanth gum. The small plaque types bred true after cloning. A variety of viral mutants which require polycations in order to infect cells have been reported (Takemoto, 1966). Since the SP virus reported in the present study was readily plaqued in CHSE-

214 cells without the use of a polycation, the two small plaque forms may be genetically distinct.

A mixture of large and small plaques was also noted by Engleking and Leong (1981) in IHNV produced by persistently infected CHSE-214 cells maintained at 22°C. The virus displayed varying degrees of temperature sensitivity at different passage levels. However, the small plaque-forming virus did not appear to be temperature sensitive. Evidence was noted for the presence of defective interfering particles. The complexity of the IHNV population produced by the persistently infected cell line is similar to that reported for cell lines persistently infected with another rhabdovirus, vesicular stomatitis virus (Youngner et al., 1976; Holland and Villareal, 1974). The LP and SP virus described in the present study were derived from lytic infection of CHSE-214 cells at 16°C. No evidence of defective interfering particles was noted when SP and LP were titered by $TCID_{50}$ assay. Furthermore, SP was found to resemble the wild type IHNV whereas the stock IHNV used to initiate the persistent infection was composed of large plaque forms (Engelking and Leong, 1981). It has not yet been determined whether SP and the small plaque IHNV from the persistent infection are the same.

The numerical dominance of the small plaque IHNV *in vivo* may be a result of *in vivo* conditions of temperature and pH. The physiological pH of mammals is regulated at 7.4, but the pH of poikilothermic vertebrates is temperature dependent (Albers, 1970). The arterial blood pH of rainbow trout varies inversely with temperature, declining from 8.2 to 7.8 as the temperature rises from 4 to 20°C (Randall and Cameron, 1973). At temperatures below 15°C, the pH of the host may be in a range favorable to replication of the small plaque forming virus. Increased water temperature has been reported to reduce mortality caused by IHNV (Rucker et al., 1953; Watson et al., 1954; Ross et al., 1960; McAllister, 1973; Wingfield and Chan, 1970; Amend, 1970; Amend, 1976). A possible mechanism for this effect may be the decrease in pH of the host to levels inhibitory for IHNV replication. The decreased mortality cannot be attributed to temperature sensitivity of the virus, because IHNV is routinely cultivated at 16-18°C (Pilcher and Fryer, 1980), while a temperature of 17°C was found to be protective in sockeye salmon (Amend, 1970). Infected fish subjected to increased water temperatures are suspected to become chronic or latent virus carriers (Amend, 1970). The state of viral infection in latent IHNV carriers is not known. A form of IHNV which is relatively insensitive to temperature-induced changes in the pH of the host could

act to maintain infection during periods of elevated temperature, particularly if such a form of IHNV was also attenuated. The large plaque and the pH-insensitive small plaque forms of IHNV described in this report are candidates for such a role.

SUMMARY

1. Isolates of IHNV from different geographical areas differed in virulence for a particular salmonid species. The TR, FE and ER were more virulent for juvenile chinook salmon than RBA and AK. An unidentified but virus isolate-determined factor may determine virulence for a particular species.

2. Isolates of IHNV avirulent for one species of salmonid fish may be virulent for another. The RBA and AK isolates are highly virulent for juvenile sockeye salmon and steelhead trout, but not juvenile chinook salmon. A host factor may determine comparative susceptibility to different IHNV isolates.

3. The experimentally determined virulence of five virus isolates for chinook salmon was consistent with the history of IHNV epizootics at the location of virus isolation.

4. Moderate mortality was observed after coho salmon fingerlings were exposed to high concentrations of IHNV. Virus was recovered from about 50% of dead fish sampled. This is the first report that coho salmon may be susceptible to IHNV infection under experimental conditions.

5. Using the LD_{50} of TR as an index of susceptibility, salmonid species tested were ranked for resistance in this decreasing order: coho salmon, chinook salmon, steelhead trout and sockeye salmon.

6. The comparatively high resistance of coho salmon to IHNV was genetically transferred via sperm to rainbow trout eggs. Coho-rainbow hybrids were more resistant to TR than rainbow trout.

7. Isolates of IHNV differed in their efficiency of plaquing on cell cultures derived from a single salmonid species. In addition, plaquing efficiency of an isolate varied according to which host species was the source of the cells used in the plaque assay. Thus, the virus-specific and host specific factors can be demonstrated at the cellular level. The relative efficiency of plaquing of an isolate on cell lines from different species was in agreement with historical records of species affected by the isolate and with the comparative virulence of the isolate for different salmonid species.

8. The species tropisms of IHNV isolates were also shown by comparing the $TCID_{50}$ of isolates when titered by the endpoint dilution method using CHSE-214, SSE-5 and STE-137 cells.

9. Cytopathic effect was observed when TR and AK were titered in CSE-119 cells. The final titers of the two virus isolates were much lower than the titers in CHSE-214, SSE-5 or STE-137 cells. Cytopathic effect was also observed when TR was used to infect CSE-119 cells in a multicyclic growth experiment. The amount of virus produced in CSE-119 cells was much lower than the amount produced in CHSE-214 cells. The relative resistance of CSE-119 cells is in agreement with the relative resistance of coho salmon to IHNV.

10. The plaque size of four IHNV isolates was compared at the two-passage level using agarose overlay of CHSE-214 cells at 16^{o}C. A notable proportion (35.5%) of ER formed large plaques. Less than 5% of TR, RBA and AK plaques were large.

11. Only small plaques were found in unpassaged virus from adult and juvenile trout. When a mixture of large and small plaques was used to infect juvenile chinook salmon, unpassaged virus from the resultant mortalities yielded a mixture greatly enriched in small plaques. Thus the small plaque appears to predominate *in vivo* and may represent the wild type plaque morphology.

12. Attempts to clone the large and small plaques were partially successful. High titer preparations containing 83.2-89% large and 89.6-98.5% small plaques were obtained by four cycles of picking plaques and growing cloned virus at 16°C.

13. The mean plaque diameter of LP was significantly greater than SP-IHNV after 6 days of incubation at 16°C. This difference was increased at 8, 10 and 12 days, showing that the large and small plaque-forming viruses are two different plaque types isolated from the same virus stock.

14. The mean plaque diameter and plaquing efficiency under agarose overlay of the partially purified large and small plaque populations was determined at 6, 12, 16 and 22°C. At 6°C no plaques were observed. At 12°C, the mean diameter of the large plaque population was only slightly larger than that of the small plaque population, since both populations displayed large plaque morphology at this temperature. At 16°C the typical large and small plaque morphology of the two populations was observed. At 22°C no plaques were observed in flasks inoculated with SP virus but small plaques were observed in flasks inoculated with LP virus. The plaquing efficiency of LP virus under agarose was significantly greater at 12°C than at

16°C. The number of plaques formed by LP virus was greatly reduced at 22°C, compared to the number at 12 and 16°C. The SP virus displayed similar plaquing efficiency at 12 and 16°C. The apparent temperature sensitivity of SP virus plaque size was later shown to probably be due to inhibition by low pH.

15. The effect of pH on plaque size was determined by plaquing LP virus and SP virus under agarose overlays containing HEPES buffer at 16°C. Both the LP and SP virus exhibited large plaque size under agarose maintained at pH 8.3. Under agarose maintained at pH 7.5, LP virus produced large plaques but SP virus produced small plaques. The LP virus appears to be a form of IHNV capable of producing plaques in CHSE-214 cells under more acidic conditions than the wild type.

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