# Antigenic Relationships in a Group of Three Viruses of Salmonid Fish by Cross Neutralization[1] (35724)

B. B. McCain, J. L. Fryer, and K. S. Pilcher

*Department of Microbiology, Oregon State University, Corvallis, Oregon 97331*

Three viruses isolated from young Pacific salmon during epizootics at hatcheries have been described. One of them, the sockeye salmon virus, has been found in natural disease outbreaks of this species only (*Oncorhynchus nerka*) and many of its properties have been reported (1). The Sacramento River chinook virus has caused epizootics in young chinook salmon (*Oncorhynchus tshawytscha*) in hatcheries located in the Sacramento and Mokelumne River drainages in California. Many of its characteristics have also been determined (2). The third agent, called the virus of infectious hematopoietic necrosis, was isolated from both sockeye salmon and rainbow trout (*Salmo gairdneri*) during separate hatchery outbreaks in British Columbia (3). A number of similarities among these viruses are apparent from the published data. These include similarity in the pathological changes in diseased fish; a similar cytopathic effect in cell cultures; ether sensitivity and the presence of RNA; and a similar range of virus particle size. Bullet shaped and spheroidal particles have been reported in electron micrographs of some partially purified preparations of these viruses and presumed to represent the infectious agents (4). The purpose of the present experiments was to examine possible antigenic relationships between them by means of plaque neutralization assays with homologous and heterologous antisera.

*Materials and Methods. Cell cultures.* Two established lines of salmonid cells were employed in these experiments. The CHSE-214 line was derived from chinook salmon embryos at the eyed egg stage by a trypsinization method (5) and had been carried through about 80 transfers when this work was started. The SSE-30 line was derived from sockeye salmon embryos by the same method and had been through about 60 transfers. They were maintained as monolayer cultures in Eagle's minimum essential medium (MEM) (6) with 10% agamma calf serum (Hyland Laboratories), 100 units of penicillin, 100 $\mu$g of streptomycin, and 25 units of Mycostatin/ml. This is referred to below as the complete medium.

*Viruses.* The isolation of the Oregon strain of the sockeye salmon virus (OSV) and its maintenance in sockeye salmon cell cultures have been described previously (1). The stock virus used in the present experiments was fluid from infected cell cultures, stored at $-60°$. It contained $3.0 \times 10^6$ plaque-forming units (pfu)/ml. The Sacramento River chinook disease virus (SRCV) was isolated at the U.S. Fish and Wildlife Service Western Fish Disease Laboratory in Seattle, Washington, from tissue of a young chinook salmon during an epizootic at the Coleman National Fish Hatchery in California. The virus used in these experiments was received through the courtesy of Dr. W. H. Wingfield of the California Department of Fish and Game. It consisted of frozen fluid from infected cultures of chinook salmon embryonic cells. Stock virus was prepared by inoculating cultures of sockeye salmon cell line SSE-30 in complete medium, incubating 3 days at 18°, and harvesting the culture fluid. The fluid was centrifuged 30 min at 2200*g*, dispensed in 0.5-ml volumes in screw cap tubes, and stored at $-60°$. After thawing, the virus contained $1.8 \times 10^7$ pfu/ml. The infectious hematopoietic necrosis virus (IHN) was iso-

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lated by Amend *et al.* (3) from moribund fingerling rainbow trout (*Salmo gairdneri*) and sockeye salmon during separate hatchery epizootics in British Columbia in 1967. A specimen of this virus was received in the form of frozen fluid from infected fathead minnow cell cultures, through the courtesy of D. F. Amend, Western Fish Disease Laboratory. Stock virus was prepared in SSE-30 cell cultures in the manner described above for the SRCV. It contained $8.0 \times 10^6$ pfu/ml.

*Infectivity titrations.* The infectivity of virus preparations was titrated by either the end point dilution method or the monolayer plaque assay. In the former method cultures of SSE-30 cells in small Pyrex tubes were used. Decimal dilutions of the virus were prepared, and each dilution was inoculated in 5 of the tube cultures. They were examined for cytopathic effect after 7-days incubation at 18°. Details describing this method have been reported earlier (1). The 50% end point was estimated by the method of Reed and Muench (7), and the infectivity was recorded in terms of the number of 50% infectious doses of virus ($TCID_{50}$)/ml.

The plaque assay is described below as a part of the technique used in plaque neutralization tests. In measuring infectivity of stock virus preparations, 5-fold dilutions of the virus were made, and 3 plaque bottles were inoculated for each dilution. The result was recorded (pfu/ml).

*Preparation of antisera.* Antisera for each of the 3 viruses were prepared by injection of rabbits with a partially purified, concentrated suspension of virus emulsified in complete Freund's adjuvant (Difco). Each virus was propagated in monolayer cultures of SSE-30 cells in which the complete medium had been modified by the substitution of 0.4% bovine serum albumin for the 10% agamma calf serum. Sufficient virus was inoculated to give a concentration of about $1.5 \times 10^3$ $TCID_{50}$/ml, and cultures were incubated at 18° until 70 to 90% of the cells showed a cytopathic effect. The virus in the culture fluid was then partially purified by centrifugation at 2200*g* for 30 min, followed by sedimentation of the virus at 55,000*g* for 60 min. The virus pellet was then resuspended in a small volume of sterile MEM, estimated to give a concentration of about $10^9$ $TCID_{50}$/ml. The suspension was then emulsified with an equal volume of Freund's complete adjuvant. Rabbits to be immunized received 1 ml of the virus suspension in MEM intravenously, and two 1-ml injections of the virus emulsion intramuscularly. Two weeks later, the intramuscular injections were repeated. Three weeks after the second injections, the animals were bled for antiserum. Serum was sterilized by Millipore filtration, dispensed in small volumes in sterile tubes, and held at either 4°, or in a freezer at −60°. Serum from nonimmunized rabbits was prepared in the same manner.

*Plaque neutralization tests.* Each antiserum was assayed for neutralizing activity against each of the 3 viruses. The serum to be tested was first heated at 56° for 30 min. It was then diluted with MEM containing 0.4% bovine serum albumin, at pH 7.4, to give a series of five twofold dilutions previously shown to produce 10 to 90% plaque neutralization with the homologous virus. The serum dilutions were then dispensed in 1-ml volumes into 3 parallel sets of sterile screw cap tubes. Included with each set was a tube containing 1 ml of normal rabbit serum diluted to the same extent as the lowest antiserum dilution in the series.

A tube of one of the stock viruses was thawed and diluted in the above diluent to give approximately 100 pfu/0.15 ml. The proper dilution had been determined from a previous titration. One ml of the diluted virus was then added to each of the 5 tubes containing 1 set of serum dilutions, and the tube of diluted normal serum. After mixing the contents, the tubes were incubated at 18° for 60 min. Proper dilutions of the other 2 viruses were prepared in the same manner, and each was added to a set of the twofold serum dilutions.

The unneutralized virus in each serum virus mixture was measured by plaque assay in monolayer cultures of CHSE-214 cells. These cultures were prepared 8 to 10 days beforehand by inoculating 2-oz prescription bottles with 5 ml of a suspension containing about $5 \times 10^5$ cells/ml in complete medium,

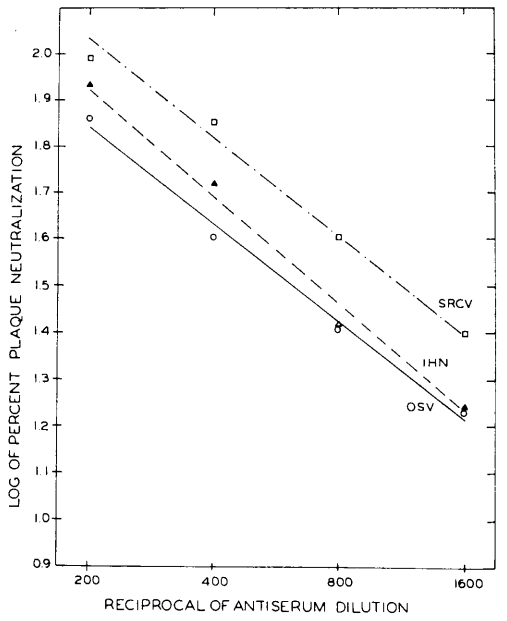


FIG. 1. Neutralization of OSV, IHN, and SRCV viruses by antiserum against OSV, as measured by reduction in numbers of plaques produced by serum virus mixtures in monolayer cultures of chinook salmon cell line CHSE-214. All points represent mean values from 2 experiments.

The medium was changed once, after 4 or 5 days. To perform the virus assay, the cell monolayers in bottles to be used were rinsed twice with 3-ml volumes of MEM. A set of 3 culture bottles was used for each serum–virus mixture, and each bottle received 0.3 ml of the mixture. The bottles were tilted in a rotary fashion to distribute the virus, and this was repeated several times during a 2-hr period allowed for adsorption of the virus at 18°. The bottles were then placed at 4° for 30 min.

An agar overlay mixture was prepared in the following way: Complete medium was made up at double the desired concentration of all components, heated to 45°, and combined with an equal volume of sterile, melted 1.5% Ionagar No. 2 (Oxoid) in double-distilled water cooled to 45°. Five ml of the overlay mixture were then distributed over the monolayer in each cooled bottle containing adsorbed virus, and the agar was allowed to solidify for 1 hr. All bottles were then inverted and incubated at 18°.

When virus plaques were apparent, usually after 4 days, the bottles were cooled at 4° for 30 min. Three ml of an agar overlay mixture prepared as above, but without serum, and containing 50 μg of neutral red/ml, were layered on top of the original overlay in each bottle. The bottles were inverted and incubated in the dark at 18° for 1 or 2 days, when plaques were counted. Bottles containing from 20 to 200 plaques were used for counts, and the mean count for the triplicate bottles receiving the same serum–virus mixture was determined. The percentage reduction in mean plaque count produced by each serum dilution was calculated by comparison of the appropriate mean plaque count with that obtained from the mixture of virus and diluted normal rabbit serum.

*Results and Discussion.* The results of plaque neutralization assays with OSV antiserum tested against OSV, IHN, and SRCV are shown in Fig. 1. It is apparent that the antiserum neutralized the infectivity of all 3 viruses. The relationship between the serum dilution and the percentage of plaque neu-

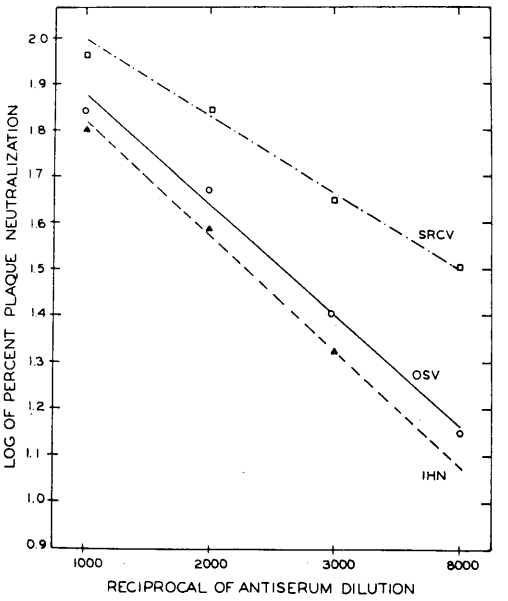


FIG. 2. Neutralization of OSV, IHN, and SRCV viruses by antiserum against SRCV, as measured by reduction in numbers of plaques produced by serum virus mixtures in monolayer cultures of chinook salmon cell line CHSE-214. All points represent mean values from 2 experiments.

TABLE I. Plaque Neutralization of OSV, IHN, and SRCV Viruses by their Homologous and Heterologous Antisera.[a]

| | Reciprocals of serum dilutions giving 50% reduction in plaques | | | | | |
|---|---|---|---|---|---|---|
| Antiserum: | OSV | | IHN | | SRCV | |
| Virus Expt.: | 1 | 2 | 1 | 2 | 1 | 2 |
| OSV | 330 | 350 | 140 | 144 | 1900 | 1460 |
| IHN | 340 | 480 | 152 | 164 | 1600 | 1330 |
| SRCV | 540 | 780 | 560 | 346 | 3900 | 3400 |

[a] The serum dilutions corresponding to 50% plaque neutralization were read from separate graphs of the data from Expts. 1 and 2, like those of Figs. 1 and 2, which are based on mean values from the 2 experiments. The least significant difference between the means of duplicate assays for serum dilutions producing an equivalent percentage of plaque neutralization was estimated from the 9 pairs of duplicate assays in Table I. Values were converted to logs and the standard error of the difference between the means of duplicate assays was estimated (9). The least significant difference between such means was found to be a difference greater than 0.1816 log units.

tralization observed in each case appears to be approximately linear within the range of dilutions employed, and provided that both variables are plotted logarithmically. This type of relationship has been reported with other virus antibody systems (8). The data obtained for the OSV and IHN viruses are not significantly different. However, the neutralization line for the SRCV is at some distance from the other 2 lines, and it was found that the serum dilution values for most points on this line were significantly different from parallel points on the OSV line.[2] It was somewhat unexpected to find that the SRCV was neutralized by higher dilutions of antiserum than those giving an equivalent effect on the OSV, for which the serum was homologous.

Figure 2 presents the results of plaque neutralization assays with SRCV antiserum tested against the same 3 viruses. Here again the serum neutralized the infectivity of all 3 agents. The antibody content was higher than that of the OSV antiserum in Fig. 1. In this case also, the lines for the OSV and IHN were not significantly different. However, the serum dilution values for all points on the SRCV line were significantly different from parallel points on the other 2 lines. Hence in this case also the SRCV was neutralized by higher serum dilutions than those giving equivalent neutralization of the other agents.

[2] See footnote *a*, Table I.

In the plaque neutralization test the titer of an antiserum is usually expressed as the highest dilution that reduces the plaque count by 50%, or 80%. In Table I, the results of plaque neutralization assays with OSV antiserum, IHN antiserum, and SRCV antiserum tested against each of the 3 viruses are presented in terms of the serum dilutions giving 50% plaque neutralization. In the case of the OSV and SRCV antisera, the data were taken from the same assays which are graphed in Figs. 1 and 2, except that the variation between 2 separate assays on the same serum virus system is shown, instead of the mean values used in Figs. 1 and 2. In addition, Table I includes results obtained with the IHN antiserum, which had a lower antibody titer than the others. Note, however, that the dilutions of this serum giving 50% plaque neutralization were very similar for the OSV and IHN viruses, while the equivalent dilution for the SRCV was significantly higher. Thus with all 3 antisera, whether homologous or heterologous, 50% plaque neutralization of the latter agent was achieved with higher serum dilutions than those required for comparable activity against the other 2 viruses.

The present results indicate that these 3 viruses of salmonid fish are antigenically related, and that insofar as this one type of analysis can determine, the OSV and IHN agents are apparently indistinguishable. It

may be noted however, as reported by Amend *et al.* (3) that differences have been observed in the natural diseases produced by these 2 viruses, with respect to symptomatology, histopathology, and mortality rates. The IHN virus has been the cause of epizootics at hatcheries in both sockeye salmon and rainbow trout; whereas natural disease due to OSV has been found only in sockeye salmon.

The data in Figs. 1 and 2, and in Table I seem to indicate that the SRCV is sufficiently different from the other 2 to be distinguishable by the plaque neutralization analysis, even though the antigenic relationship appears to be close. The finding that this agent was neutralized by higher dilutions of both homologous and heterologous antibody than in the case of the other 2 was unexpected. A hypothetical explanation may be suggested as follows: The virus itself is native to and isolated from the chinook salmon species. The cell line used in the plaque neutralization assays (CHSE-214) was originally derived from chinook salmon embryos. It seems possible that the plaque-forming unit of this virus for this cell line may represent a smaller number of virus particles than are required for a plaque-forming unit of the other 2. In this case, assuming a fairly close antigenic relationship between the viruses, it appears reasonable that a smaller number of antibody molecules would be required to neutralize 1 pfu of the SRCV than would be needed for comparable activity against the other agents.

Some differences in the species specificity of SRCV, IHN, and OSV viruses have also been observed. Natural epizootics due to SRCV have thus far been observed only in juvenile chinook salmon, though laboratory infection of chinook, sockeye, and rainbow trout has been accomplished by simple water contact (2). It has been reported, however, that chinook are refractory to experimental infection with IHN virus (3). Similarly it has been noted in our laboratory that, when SRCV was inoculated in cultures of a chinook salmon cell line (CHSE-114), moderately high titers of progeny virus and a cytopathic effect were produced in the first passage. In the same cell line, OSV produced lower titers and no cytopathic effect in the first passage.

Additional antigenic analyses of these three viruses by means of complement fixation and immunodiffusion are needed to provide a more complete understanding of their relationships.

*Summary.* Three viruses of salmonid fish, the Oregon sockeye salmon virus (OSV), the Sacramento River chinook salmon virus (SRCV), and the infectious hematopoietic virus of sockeye salmon and rainbow trout (IHN), have been studied by means of the plaque neutralization technique, with both homologous and heterologous antisera. The OSV and IHN agents were indistinguishable by this method. The SRCV was closely related to the other 2 antigenically, but the results of the cross neutralization assays indicated a significant difference between this virus and the others.

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