Comparison of in vitro Growth Characteristics of Ten Isolates of Infectious Haematopoietic Necrosis Virus

By D. MULCAHY,* R. PASCHO AND C. K. JENES
United States Fish and Wildlife Service, National Fishery Research Center, Building 204, Naval Station, Seattle, Washington 98115, U.S.A.
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SUMMARY

Ten isolates of infectious haematopoietic necrosis from salmonid fishes of different locations on the West Coast of North America from California to Alaska were compared by plaque size, single-step growth curves at 15 and 18 °C, rate of appearance of cytopathic effects in cell cultures, and growth over a range of temperatures. All isolates were distinguishable on the basis of each growth characteristic examined. The CO isolate from the Sacramento River drainage of California was the most singular of the 10 because of its diminutive plaque size and sensitivity to slightly elevated temperatures. The mean plaque diameter of the 10 isolates increased as the latitude of the geographic source of the isolate increased. Although the maximum titre obtained by all isolates was depressed at temperatures above approximately 18 °C, half of the isolates were not inhibited by temperatures as low as 0·5 °C.

INTRODUCTION

Infectious haematopoietic necrosis, a virus disease of salmonid fishes, was initially believed to be several related but distinct diseases. It was first recognized in the 1950's in sockeye salmon (Oncorhynchus nerka) hatcheries in Washington and Oregon on the West Coast of the United States and became known as sockeye salmon virus disease (Rucker et al., 1953; Watson, 1953; Wingfield et al., 1969). A similar virus disease known as Sacramento River chinook disease was reported in chinook salmon (O. tshawytscha) in California (Ross et al., 1960; Parisot & Pelnar, 1962). Amend et al. (1969) isolated a virus from moribund rainbow trout (Salmo gairdneri) and sockeye salmon in British Columbia, Canada, and named the condition infectious haematopoietic necrosis (IHN) virus disease. These early descriptions distinguished the three diseases on the basis of host and geographic range. The three diseases have been grouped under the common name of IHN because of similarities in clinical signs (Amend et al., 1969), pathology (Yasutake, 1970), serology (McCain et al., 1971), and biological and physical characteristics (Amend & Chambers, 1970). The IHN virus is distinct from other fish rhabdoviruses in ultrastructure (Cohen & Lenoir, 1974), serology (Vestergard Jorgensen, 1972; McAllister et al., 1974a), protein structure (McAllister & Wagner, 1975; Lenoir & De Kinkelin, 1975), and associated clinical signs and histopathology (Yasutake, 1975).

Although IHN is now considered to be a single disease, the early distinctions between sockeye salmon virus disease, Sacramento River chinook disease and IHN suggested that different strains of the virus might exist. Preliminary observations of IHN virus isolates from hatchery and wild fish revealed variations in plaque morphology and optimum growth temperature. We compared the in vitro growth characteristics of 10 isolates of IHN virus from different geographic locations to determine the extent to which strain differences exist.

METHODS

Viral isolates and cell cultures. Ten isolates of IHN virus from salmonid fishes of the Pacific Coast of the United States (identified in Table 1) were studied. Serum neutralization tests confirmed that all isolates were IHN virus. Although various cell lines had been used for initial isolation of the isolates, we used only the epithelioma papulosum cyprini (EPC) cell line. Stock cells were grown in Eagle's minimum essential medium with Earle's salts (MEM, Autopow, Flow Laboratories) containing 10% foetal bovine serum (MEM-10). In making dilutions,
Table 1. Isolates of IHN virus used for comparison of growth characteristics

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Location</th>
<th>Species and sample type</th>
<th>Year isolated</th>
<th>Cell line passages</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>Coleman National Fish Hatchery, Anderson, California</td>
<td>Chinook salmon moribund fingerling</td>
<td>1980</td>
<td>EPC-2</td>
</tr>
<tr>
<td>TH</td>
<td>Trinity Hatchery, Eureka, California</td>
<td>Chinook salmon ovarian fluid</td>
<td>1980</td>
<td>EPC-3</td>
</tr>
<tr>
<td>ER†</td>
<td>Elk River Hatchery, Grants Pass, Oregon</td>
<td>Chinook salmon ovarian fluid</td>
<td>1979</td>
<td>Other &lt; 5</td>
</tr>
<tr>
<td>WS‡</td>
<td>Warm Springs Hatchery, Madras, Oregon</td>
<td>Steelhead trout ovarian fluid</td>
<td>1980</td>
<td>EPC-4</td>
</tr>
<tr>
<td>NS‡</td>
<td>Nan-Scott Lake, Salem, Oregon</td>
<td>Kokanee salmon kidney, spleen pool</td>
<td>1971</td>
<td>Other &gt; 10</td>
</tr>
<tr>
<td>SU†</td>
<td>Suttle Lake, Bend, Oregon</td>
<td>Kokanee salmon ovarian fluid</td>
<td>1980</td>
<td>Other &lt; 5</td>
</tr>
<tr>
<td>TA†</td>
<td>Tamgas Creek, Annette Island, Alaska</td>
<td>Pink salmon ovarian fluid</td>
<td>1981</td>
<td>EPC-2</td>
</tr>
<tr>
<td>LT</td>
<td>Lower Thumb River, Karluk Lake, Kodiak Island, Alaska</td>
<td>Sockeye salmon ovarian fluid</td>
<td>1979</td>
<td>EPC-3</td>
</tr>
</tbody>
</table>

* Isolates were made by the authors unless otherwise indicated.
† Provided by Dr J. Leong, Oregon State University, Corvallis, Or., U.S.A. The TA isolate was originally made by Mr J. Cvitanich, Oregon Aqua-Foods, Springfield, Or., U.S.A.
‡ Provided by Mr S. Leek, U.S. Fish and Wildlife Service, Cook, Wash., U.S.A.
§ Provided by Mr K. Amos, Washington Department of Fisheries, Olympia, Wash., U.S.A.

MEM buffered with Tris (MEM-Tris) was used. Cell cultures incubated in ambient atmosphere systems were grown in MEM-Tris containing 5% foetal calf serum (MEM-5-Tris).

Effect of cell seeding densities on plaque diameter. The standard plaque titration method developed for IHN virus was used (Burke & Mulcahy, 1980). One-month-old EPC cells were suspended in MEM-10, counted, and diluted to densities of 1.0 × 10⁶, 1.5 × 10⁶, 2.0 x 10⁶ and 2.5 x 10⁶ cells/ml. Each cell suspension (2 ml/plate) was added to each of 50 35 mm cell culture dishes and incubated for 18 h at 20 °C. Then, each plate received 0.1 ml of the CR isolate of IHN virus diluted to give approximately 30 plaques. After 1 h, nutrient overlay was added and the plates were incubated at 15 °C for 7 days.

To measure plaque diameters, an inverted microscope equipped with a calibrated eyepiece micrometer was used. A total of 258 discrete plaques were measured at each cell density, and the mean and standard deviation of plaque sizes were determined.

Effect of incubation time on plaque size. The CO and CR isolates (Table 1) were used to standardize the incubation time for comparison of plaque diameters. Twenty plates of each isolate were fixed and stained after 7, 8, 9 and 10 days of incubation and the diameters of 275 plaques measured. Mean diameters and standard deviations were calculated.

Comparison of plaque sizes. The mean plaque diameters of all virus isolates were determined in a single experiment. Inoculated plates (20 per isolate) were fixed and stained after 10 days of incubation. The diameters of 350 well-separated plaques of each isolate were measured and the mean diameters and standard deviations calculated.

Single-cycle growth curves. Single-cycle growth curves of released and cell-associated virus were determined at 15 and 18 °C in a procedure modified from that of Rovozzo & Burke (1973). Monolayers of EPC cells were grown in 35 mm Petri plates at 25 °C for 18 h; the medium was then removed and the cells were inoculated with 0.2 ml of virus diluted to yield an m.o.i. of at least 4. After a 60 min adsorption period, the cells were washed three times with MEM-Tris to remove unattached virus, and 2 ml of MEM-5-Tris precooled to 15 or 18 °C were then added to the plates. Two plates from both the 15 °C and 18 °C groups were immediately removed to determine the initial (T₀) virus concentrations. The remaining plates were placed at 15 or 18 °C. At various intervals up to 189 h, two plates were removed from the 15 and 18 °C groups for viral titration.

At each sampling time we examined the cell monolayers for cytopathic effect (c.p.e.). The supernatants were removed from the plates and placed at 4 °C, and the cell layers were washed twice with MEM-Tris. Few detached cells were seen before 20 h. Supernatants and washes collected from samples taken after 20 h of incubation were centrifuged (2000 g) to recover detached cells. The pellet cells were resuspended in 2 ml MEM-5-Tris and returned to the original plates. The plates were twice frozen at - 70 °C and rapidly thawed. The medium was removed and centrifuged (2000 g) to remove cell debris. The level of released and cell-associated viruses was titrated...
by the plaque method described above. Total virus production was calculated by adding the titres of the released and cell-associated virus.

*Stages of development of c.p.e.* Before the medium and cells in the single cycle were harvested for virus titration, the cell cultures in the growth curve experiments were examined with an inverted microscope. The extent of c.p.e. was subjectively scored as follows: 1+, scattered foci of slightly rounded and refractile cells; 2+, obvious foci of rounded cells ('grape clusters') with a few detached cells, foci separated by apparently normal cells; 3+, widespread c.p.e., extensive cell detachment; 4+, disintegration of the cell sheet, most cells floating free, few (if any) normal cells.

*Virus and cell growth across a temperature gradient.* Growth of the IHN virus isolates and EPC cells across a wide range of temperatures was studied by using a temperature gradient incubator (Model TN-3, Scientific Industries, Inc., Bohemia, N.Y., U.S.A.). The incubator was equilibrated for at least 2 days before each experiment and 18 temperatures from 0-5 °C to 25 °C were selected. Most temperatures were between 13 °C and 24 °C, to enable us to define accurately the curve of decreasing virus titre with increasing temperature. The temperatures used for each experiment varied slightly because the gradient could not be reproduced exactly.

A suspension of EPC cells (2 ml of 10⁶ cells/ml) was added to individual tissue culture tubes (No. 25200, Corning) and incubated for 18 h at 25 °C. The number of cells per tube was then calculated to determine the starting cell count for the cell growth experiment and to determine the virus dilution necessary to give an m.o.i. of 4 for the viral growth curves. The medium was decanted, 1 ml of diluted virus was added to each tube, and viral adsorption continued for 1 h at 20 °C. The cells were washed twice with 5 ml MEM-Tris, and then 2 ml MEM-10 were added to each tube. Two tubes were placed in the incubator at each selected temperature. Two tubes were used immediately to determine the initial virus titre.

Tubes were removed after 7 days and the cells examined for c.p.e. The level of released virus was determined in the tubes showing complete cell destruction (4+). The remaining tubes were returned to the incubator until 14 days post-infection, when the virus was titrated regardless of the stage of c.p.e. Virus concentration was plotted against temperature; levels of virus above that present at T₀ were considered to represent viral growth. The amount of virus produced per cell was determined by dividing the mean viral titre obtained at each temperature by the initial cell concentration. The resulting viral yields per cell for each isolate were plotted against temperature.

Growth of the EPC cell line over the temperature gradient was determined in two experiments. The procedure was the same as previously described except that no virus was added to the cell cultures and all cultures were removed after 7 days. The mean number of cells at each temperature was determined from cell counts on two tubes.

RESULTS

*Plaque morphology*

The IHN virus isolates examined formed very small, clear plaques with an average diameter of 800 µm after 10 days. Microscopically, cell debris could be seen in the centre of the plaques, but living cells were never present. The cells at the periphery of the plaque were rounded and clustered, but little or no c.p.e. could be seen in the cells beyond the immediate plaque margin. Some elongated cells extended from the plaque margin towards its centre. Some variation in plaque size was evident with each isolate. Efforts to pass small and large plaque mutants that bred true were unsuccessful.

*Effect of cell seeding density on plaque diameter*

The maximum mean plaque diameter (486 ± 68 µm) occurred when 1.5 × 10⁶ cells/ml were used to seed the culture plates. When 2.5 × 10⁶ cells/ml were used, the mean plaque diameter was reduced to 437 ± 69 µm. The pH of the growth medium dropped more quickly in the plates seeded at the two highest cell densities than in the plates seeded with fewer cells, as indicated by the indicator dye in the medium. A cell density of between 1.0 × 10⁶ and 1.5 × 10⁶ cells/ml was used for the rest of the study.

*Effect of incubation time on plaque diameter*

Although CR isolate plaques were clearly visible and easily measured after 7 days of incubation, CO isolate plaques were tiny and irregular, with many foci of infection visible only by microscopy. The CO isolate plaques did not begin to appear until the eighth day of incubation. The mean plaque diameters of both isolates increased with time (Table 2), attaining a maximum difference in mean diameters after 10 days of incubation, the incubation time selected for all further comparisons of plaque size.
Table 2. Effects of days of incubation at 15 °C on mean plaque diameters of the CO and CR isolates*

<table>
<thead>
<tr>
<th>Days of incubation</th>
<th>Mean plaque diameters (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO</td>
</tr>
<tr>
<td>7</td>
<td>367 (±72)</td>
</tr>
<tr>
<td>8</td>
<td>447 (±76)</td>
</tr>
<tr>
<td>9</td>
<td>564 (±92)</td>
</tr>
<tr>
<td>10</td>
<td>681 (±93)</td>
</tr>
</tbody>
</table>

* The results were calculated from the measurement of 275 discrete plaques; standard deviations are shown in parentheses.

Table 3. Time of development of c.p.e. at two temperatures, temperature ranges allowing growth and mean plaque diameters*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Time (h) of first appearance of c.p.e.</th>
<th>Time (h) of complete c.p.e.</th>
<th>Optimal temperature range (°C)</th>
<th>Upper temperature limit for growth (°C)</th>
<th>Mean plaque diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 °C</td>
<td>18 °C</td>
<td>15 °C</td>
<td>18 °C</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>33</td>
<td>25</td>
<td>81</td>
<td>189</td>
<td>9.8-11.5</td>
</tr>
<tr>
<td>ER</td>
<td>33</td>
<td>25</td>
<td>81</td>
<td>81</td>
<td>6.5-15.5</td>
</tr>
<tr>
<td>TH</td>
<td>57</td>
<td>57</td>
<td>105</td>
<td>93</td>
<td>3.9-13.5</td>
</tr>
<tr>
<td>WS</td>
<td>45</td>
<td>45</td>
<td>81</td>
<td>117</td>
<td>4.2-17.0</td>
</tr>
<tr>
<td>NS</td>
<td>25</td>
<td>20</td>
<td>69</td>
<td>57</td>
<td>3.5-18.0</td>
</tr>
<tr>
<td>SU</td>
<td>33</td>
<td>20</td>
<td>81</td>
<td>69</td>
<td>4.0-15.5</td>
</tr>
<tr>
<td>CR</td>
<td>25</td>
<td>25</td>
<td>57</td>
<td>57</td>
<td>13.5-16.5</td>
</tr>
<tr>
<td>TA</td>
<td>33</td>
<td>33</td>
<td>189</td>
<td>189</td>
<td>13.4-14.0</td>
</tr>
<tr>
<td>LT</td>
<td>25</td>
<td>25</td>
<td>117</td>
<td>93</td>
<td>4.8-15.5</td>
</tr>
<tr>
<td>LR</td>
<td>ND‡</td>
<td>ND‡</td>
<td>ND‡</td>
<td>ND‡</td>
<td>3.0-17.0</td>
</tr>
</tbody>
</table>

* The results were determined after 10 days of incubation at 15°C and calculated from the measurement of 350 individual plaques of 10 IHN virus isolates; standard deviations are shown in parentheses.
‡ ND, Not done.

Comparison of plaque sizes of IHN virus strains

Histograms of the plaque diameter measurements showed the distributions to be generally bell-shaped. The CO isolate formed the smallest plaques (X̄ = 625 μm), and the LT isolate the largest (X̄ = 985 μm) plaques (Table 3). The rest of the isolates examined could not be distinguished immediately on the basis of plaque size.

In general, mean plaque diameters increased with the increasing latitude of the sites from which the isolates originated (Table 3). The isolates with the smallest plaques (< 750 μm) were from California and southern Oregon (CO, ER, TH), and those with the largest (> 900 μm) were from Alaska (LT); isolates with intermediate-size plaques (750 to 900 μm) were from central Oregon and Washington (WS, NS, SU, LR, CR). An exception was the TA isolate (X̄ = 811 μm), which had a mean diameter closer to those of the Oregon-Washington isolates, although it came from fish of southeastern Alaska.

Growth curves

Growth of the CO isolate at 18 °C was strongly depressed (>1 log₁₀ difference) compared with growth at 15 °C (Fig. 1). The TA and TH isolates also showed decreased growth at 18 °C but the difference was less (about 0.5 log₁₀ difference) than that for the CO isolate.

The lag periods (average 10 h) ranged from about 5 h for the CR isolate to about 20 h for the WS isolate, and overall were slightly shorter at 18 °C than at 15 °C. Cell-associated and released viral levels corresponded closely in the early stages of growth, but the cell-associated virus dropped below the level of released virus as viral production peaked. The TA and WS isolates were exceptions. Maximum viral production varied with the isolate and the temperature, ranging from about 30 h at 18 °C for the SU and NS isolates to 90 h at 15 °C for the LT isolate.
Fig. 1. Single-cycle growth curves of nine IHN virus isolates at 15 °C (○) and 18 °C (●) showing total (released and cell-associated) virus. LR isolate was omitted.

Development of c.p.e.

The time at which c.p.e. was first noticeable varied between isolates and between the two incubation temperatures (Table 3). The rate of development of c.p.e. was not related to the time of its first appearance. At 15 °C, c.p.e. was first visible at 25 h post-infection for isolates NS, CR and LT; at 18 °C, the earliest c.p.e. appeared at 20 h post-infection for the NS and SU isolates.

Usually, c.p.e. progressed similarly for cultures held at 15 °C and 18 °C, but somewhat faster at 18 °C than at 15 °C (Table 3). However, for both the WS and CO isolates development of maximum c.p.e. was much slower at 18 °C than at 15 °C. For the CO isolate, maximum c.p.e. at 18 °C did not occur until the final sampling period (189 h). Maximum c.p.e. developed most quickly for the CR isolate (57 h at both temperatures), and most slowly for the CO isolate (189 h...
Replication of IHN virus and EPC cells across a temperature gradient

The isolates studied grew well from about 6 to 16 °C. One exception was the CO isolate, which did not attain maximum titre above 12 °C. For all isolates, the amount of virus produced at the lowest temperatures was at least slightly reduced compared to that at higher temperatures. The CO, NS, SU, LR and LT isolates grew to nearly maximum levels at temperatures as low as 0·5 °C (Fig. 2), whereas the TH, WS, CR and TA isolates were strongly inhibited at low temperatures. The ER isolate was intermediate in behaviour. Each isolate had a range of temperatures for opti-
Isolates of IHN virus

The yield of virus per cell across the temperature gradient varied greatly among isolates (Fig. 3). Maximum yields ranged from 61 p.f.u./ml for the CR isolate to 2.4 p.f.u./ml for the CO isolate. In general, the yields per cell over the full range of temperatures were highest for the CR, SU and WS isolates. The yields per cell were highest at temperatures below 10 °C for the SU, WS, TH, ER, NS and LT isolates, and at temperatures above 10 °C for the CR, LR and TA isolates. The viral yields for the CO isolate were so low that trends across the temperature gradient were difficult to assess.

There was no increase in cell numbers at temperatures below 10 °C after the 7 day incubation period. Cell division may have occurred, but growth was not sufficient to compensate for the death rate. The increase in apparent cell numbers continued linearly to its greatest point at 25 °C, the highest temperature used in these experiments, but a temperature that is not permissive for IHN virus growth. Decreases in virus production at the higher temperatures tested were not due to inhibition of growth of the fish cell line used in this study.

**DISCUSSION**

Considerable variation was found in the *in vitro* growth characteristics of the 10 IHN virus isolates examined, and no two isolates appeared to be identical, suggesting the existence of true
strains of IHN virus. Differences between the isolates were especially noticeable in plaque diameter after 10 days incubation, and in the presence and degree of inhibition of growth at high and low temperatures. The significant variation between isolates makes it imperative that investigators clearly identify the origin and designation of the IHN virus strain used in their work.

The diameter of plaques formed by any isolate of IHN virus is sensitive to procedural variables (Burke & Mulcahy, 1980), making it necessary to compare all isolates simultaneously. Although a 7 day incubation is standard in our laboratory, we used a 10 day incubation period for the comparison of plaque diameters of the various isolates to widen the interval between the mean plaque diameters and enable us to measure the smallest plaques formed more accurately.

The mean plaque diameter of the isolates showed a direct relation to the geographic origin of the strain. As the latitude increased, the mean plaque diameters increased. Although this relation suggests a faster replication rate for the northern isolates, no correlated increase in replication rate was seen in single-cycle growth curves at 15 °C compared with 18 °C, or in low temperature growth rates.

Additional factors not considered in this study may also affect plaque size. For example, the NS isolate had a quicker growth curve than the LT isolate, produced maximum c.p.e. sooner and yielded more p.f.u./ml/cell at 15 °C but had a smaller mean plaque size. The IHN virus isolates compared in this study may induce different levels of interferon production by host cells, or they may vary in their production of defective interfering particles. When possible, IHN virus isolates with a low number of cell culture passages were used in this study to minimize production of defective interfering particles.

The CO isolate was unique among the 10 IHN virus isolates in having the smallest plaque diameter and the greatest depression in growth at elevated temperatures. Several other isolates, especially the TA isolate, grew slightly slower at 18 °C than at 15 °C, but the effect was not as pronounced as for the CO isolate. A slight serological difference was noted for an earlier isolate of IHN virus from this location (McCain et al., 1971). The partial control of IHN in Sacramento River chinook salmon hatcheries achieved by elevating the water temperature to 18 °C is a practical demonstration of the temperature sensitivity of the CO strain (Ross et al., 1960; Parisot & Pelnar, 1962). Elevation of water temperature was ineffective when other IHN virus strains were involved (Hetrick et al., 1979), unless the temperature was raised to 20 °C (Watson et al., 1954; Amend, 1970), an unsuitable temperature for rearing salmon. It is not likely that disease caused by any isolate other than CO could be controlled on a practical basis by raising the water temperature.

The IHN virus isolates differed in their rates of growth and maximum levels obtained in the single-cycle growth curves. The curves for two Oregon isolates (SU and NS) resembled those obtained for an earlier Oregon sockeye salmon isolate (McAllister et al., 1974b). Because variations in growth rates and virus yields could be detected when the isolates were grown at a single temperature, it would be of interest to examine the isolates further for differences in virulence which might be associated with growth rates or virus yield per cell.

In the test of viral replication across a temperature gradient, half of the isolates grew to near maximum levels at temperatures as low as 0.5 °C; growth of the rest was moderately to severely reduced at the lower temperatures. The ability to grow to a maximum level at low temperature appears to be a true strain difference and was not due to an increase in the number of cells available for replication. The optimum growth temperature for the EPC cell line is 30 °C (De Kinkelin & Le Berre, 1978), and in our studies cell numbers did not increase below about 10 °C. In addition, the cells were inoculated with virus at a high m.o.i. which should have precluded cell replication. The mechanism by which some isolates grow to a maximum level at low temperature is not apparent.

The differences between the IHN virus isolates originating from a range of geographic locations and host species have epizootiologicat implications. The variations in virus growth characteristics could help to determine the source of virus isolated from epizootics in hatchery fish, especially when supported by records of fish transportation. However, we do not know how fast IHN virus develops these variations, especially when influenced by changes in host species and environment. If IHN virus is inherently variable in its characteristics, highly sensitive tech-
niques such as cross-neutralization tests and protein and nucleic acid analyses may be of less value than the methods used here for epizootiological studies and for the comparison of isolates. The more sensitive procedures would tend to show that most isolates differ from one another. Some preliminary results of protein comparisons of IHN virus isolates were recently published (Leong et al., 1981).

REFERENCES


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