Key words: reoviruses/fish/shellfish

Morphological and Biochemical Properties of Four Members of a Novel Group of Reoviruses Isolated from Aquatic Animals

By J. R. WINTON,1 * C. N. LANNAN,1 J. L. FRYER,1 R. P. HEDRICK,2 T. R. MEYERS,3 J. A. PLUMB,4 AND T. YAMAMOTO5

1Department of Microbiology, Oregon State University, Corvallis, Oregon 97331, U.S.A., 2Department of Medicine, School of Veterinary Medicine, University of California, Davis, California 95616, U.S.A., 3Alaska Department of Fish and Game, P.O. Box 3-2000, Juneau, Alaska 99802, U.S.A., 4Department of Fisheries and Allied Aquaculture, Auburn University, Auburn, Alabama 36830, U.S.A. and 5Department of Microbiology, University of Alberta, Edmonton, Alberta T6G 2E9, Canada

(Accepted 1 October 1986)

SUMMARY

The morphological, biochemical and growth characteristics of four members of the Reoviridae, three from the fish hosts, golden shiner (Notemigonus crysoleucas), chum salmon (Oncorhynchus keta) and channel catfish (Ictalurus punctatus) and one from American oyster (Crassostrea virginica), were compared. Electron microscopy of negatively stained virions revealed icosahedral particles approximately 75 nm in diameter composed of a double capsid. Complete particles had buoyant densities in CsCl of 1.34 to 1.36 g/ml. The viruses replicated well in several fish cell lines, forming plaque-like syncytia in monolayer cultures. Each virus could be distinguished by the range of cell lines supporting its growth. Polyacrylamide gel electrophoresis showed that the genome of each virus was composed of 11 segments of dsRNA distributed among three size classes. There were three large, three medium and five small segments in each genome and each isolate had a unique electropherotype. The segments ranged from 2.5 × 10^6 to 0.31 × 10^6 mol. wt. with a total genome of approximately 15 × 10^6 mol. wt. Analysis by SDS–PAGE revealed that each virus had five major structural proteins. There were two large polypeptides of approximately 135000 and 125000 mol. wt., one medium size polypeptide of 70000 mol. wt. and two small polypeptides of 45000 and 34000 mol. wt. Of the major structural proteins, those of approximately 70000 and 34000 mol. wt. were consistently present in the highest concentrations. Minor virion proteins were detected but were not characterized. These four viruses, isolated from aquatic animals, were unlike viruses of the six established genera of the Reoviridae.

INTRODUCTION

Most of the major families of animal viruses are represented among the more than 50 fish viruses either isolated in cell culture or observed by electron microscopy (Wolf & Mann, 1980; Wolf, 1984). These viruses range in importance from orphan viruses to virulent pathogens causing large losses (McAllister, 1979; Pilcher & Fryer, 1980). We have compared certain morphological and biochemical features of four reo-like viruses isolated from fish and shellfish. The viruses were unlike other members of the family Reoviridae.

The golden shiner (Notemigonus crysoleucas) virus (GSV; Plumb et al., 1979) was isolated in 1977 from moribund bait fish. Electron microscopy showed icosahedral virions approximately 70 nm in diameter. The virus was ether- and heat-resistant, stable at pH 3, 7 and 10, and remained infectious following RNase or DNase treatment. Acriflavin staining and viral replication in the presence of iododeoxyuridine suggested the agent had a dsRNA genome (Plumb et al., 1979). The virus had an optimal replication temperature of 30 °C where the latent period was 8 h and the yield was 113 infectious units per cell (Schwedler & Plumb, 1982a).
Cross-neutralization tests using GSV and infectious pancreatic necrosis virus (IPNV), a birnavirus of fish, showed no detectable cross-reactions (Schwedler & Plumb, 1980). Higher infection rates and viral titres were found among infected fish reared at increased density (Schwedler & Plumb, 1982b).

Meyers (1979) isolated a reo-like virus, termed 13p2, from juvenile American oysters (Crassostrea virginica). The virus was not affected by chloroform and was stable at pH 3 and 9 but not at pH 2. Iododeoxyuridine did not inhibit viral synthesis and the virus was not neutralized by antiserum against IPNV (Meyers, 1979). The 13p2 virus appeared circular or slightly angular depending on the particle orientation. Twenty peripheral capsomeres were observed around intact virions while cores contained six symmetrical projections or spikes at the apparent vertices of the inner capsid (Meyers & Hirai, 1980).

No viral replication or histological lesions were observed in oysters exposed to the 13p2 virus. However, inoculation of bluegills (Lepomis macrochirus) resulted in mortality, an increasing viral titre and hepatic lesions (Meyers, 1980). Infection of rainbow trout (Salmo gairdneri) produced a multifocal reticulo-endothelial granulomatous hepatitis (Meyers, 1983).

The chum salmon (Oncorhynchus keta) virus (CSV; Winton et al., 1981) was recovered from tissues of adult salmon. Electron microscopy revealed icosahedral particles 75 nm in diameter composed of a double capsid. Twenty peripheral capsomeres were seen around the virion. The outer capsid layer could be removed by treatment with a-chymotrypsin leaving a 50 nm subviral particle with enhanced infectivity. The virus was not affected by treatment with chloroform and was not neutralized by antiserum to IPNV. Injection of the virus into chum, chinook (O. tshawytscha) and kokanee (O. nerka) salmon and rainbow trout fry resulted in viral replication and low mortality in each species. A focal necrotizing hepatitis was observed in the chum and chinook salmon (Winton, 1981).

The genome of CSV was composed of 11 segments of dsRNA that fell into three size classes with a range of 2.5 × 10⁶ to 0.37 × 10⁶ mol. wt. Five major virion proteins and several additional minor proteins were detected using 12.5% gels (Winton et al., 1983).

The fourth reovirus used in this study was isolated from channel catfish (Amend et al., 1984) and designated catfish reovirus (CRV). Negative staining of purified virions showed typical reovirus-like particles with a double capsid layer. The virus had low virulence for channel catfish in infectivity trials.

Channel catfish reovirus was further characterized by Hedrick et al. (1984). The virions had an outer capsid of 75 nm and an inner capsid 55 nm in diameter. The virus genome was composed of 11 segments of dsRNA ranging in size from 2.5 × 10⁶ to 0.4 × 10⁶ mol. wt. Like CSV, there were three size classes with three large, three medium and five small segments. Five major and two minor polypeptides were detected. The proteins of the complete and empty virions were similar, ranging in mol. wt. from 132000 to 32000. Cross-neutralization studies using CRV, GSV, CSV and their respective antisera showed each virus was serologically distinct (Hedrick et al., 1984).

In this study, we used SDS–polyacrylamide gel electrophoresis to make side-by-side comparisons of the major virion proteins and genome segments of these four reo-like viruses from aquatic animals. We examined purified virions by electron microscopy and compared growth characteristics in selected cell lines. We also determined the buoyant densities of the particles by density gradient centrifugation. Although the viruses were similar in morphology and buoyant density, they could be distinguished by differences in their growth characteristics and in the electropherotypes of their structural proteins and genome segments. The viruses should be included within the Reoviridae, but they are unlike members of the established genera. Because they have all been isolated from aquatic animal hosts, we tentatively refer to them as aquareoviruses.

**METHODS**

*Viruses.* GSV (Plumb et al., 1979), 13p2 (Meyers, 1979), CSV (Winton et al., 1981) and CRV (Amend et al., 1984) were dilution-purified three times before use in these studies. Reovirus type 3 (Dearing strain) was provided by Dr J. V. Hallum, University of Oregon Health Sciences Center, Portland, Oregon, U.S.A.
Comparison of four aquatic reoviruses

Cell lines. The chinook salmon embryo cell line, CHSE-214 (Lannan et al., 1984) was used to propagate and assay the CSV, GSV and 13p2 isolates. The channel catfish ovary line, CCO (Bowser & Plumb, 1980) was used for CRV. Reovirus 3 was grown in the mouse cell line, L 929 (ATCC CCL 1). The CHSE-214, CCO and L cells were incubated at 21, 30 and 34 °C respectively.

The CHSE-214, fathead minnow (FHM; Gravell & Malsberger, 1965), bluegill fry (BF-2; Wolf & Quimby, 1966), chum salmon heart (CHH-1; Lannan et al., 1984) and brown bullhead (BB; Cerini & Malsberger, 1962) cell lines were tested for their ability to support replication of the four viruses. All cells were grown as monolayer cultures using minimal essential medium (MEM) with Earle's salts (Gibco) and 10% (MEM-10) foetal bovine serum (Sterile Systems, Logan, Utah, U.S.A.). For production and assay of virus, the serum content was reduced to 5% (MEM-5).

Virus propagation, labelling and assay. The viruses were propagated in cell cultures grown in 150 cm² flasks (Corning). Radioactive labelling of viral nucleic acid was achieved by incorporating 5 μCi/ml [5-3H]uridine (New England Nuclear) in the MEM-5 growth medium. Infectious virus was assayed using 96-well microplates (Corning) containing monolayers of appropriate cells. The titre was determined by 50% tissue culture infectious dose (TCID50) assay with endpoints calculated by the method of Reed & Muench (1938).

Virus replication and plaquing ability. The CHSE-214, FHM, BF-2, CHH-1 and BB cell lines were tested for their ability to support replication of each of the viruses. The viruses were passed several times in each cell line at 15, 22 and 25 °C and observed for production of c.p.e. The plaquing ability of the four viruses was determined by infecting CCO, CHH-1 and CHSE-214 cells in 50 cm² tissue culture dishes and covering the monolayers with a double overlay containing HEPES buffer and 5% serum (Wolf & Quimby, 1973). Cells were incubated at 25 °C for CCO and 22 °C for the CHSE-214 and CHH-1 lines. After 5 to 6 days, the cell sheets were fixed with 10% formalin and stained with 1% crystal violet.

Virus purification. When c.p.e. was extensive, any remaining cells were scraped from the surface of the flask into the growth medium and the culture fluid was centrifuged for 1 h at 100 000 g in an SW28 rotor (Beckman). The pellet was resuspended in 2 ml SSC buffer (0.15 m-sodium chloride, 0.015 m-sodium citrate, pH 7.4) and homogenized with 20 strokes of a Ten Broek homogenizer. Genetron (1,1,2-trichloro-1,2,2-trifluoroethane; Aldrich Chemicals) was added to the homogenate and mixed for 5 min. After centrifugation for 10 min at 10 000 g, the aqueous phase was removed, placed onto a continuous sucrose gradient (15 to 50% in SSC) and centrifuged for 2 h at 100 000 g in an SW28 rotor. The viral band in the centre of this gradient was removed with a probe and polyacrylamide gels (Laemmli, 1970). The dsRNA was extracted from purified CSV, GSV, CRV, 13p2 and peristaltic pump (Buchler) and pelleted by centrifugation for 1 h at 115 000 g in an SW50.1 rotor (Beckman). The pellet of partially purified virus was resuspended in 0.5 ml of SSC, layered onto a three-step CsCl gradient (2.0 ml 30%, 1.5 ml 30% and 1.0 ml 20% CsCl in SSC) and centrifuged for 16 h at 115 000 g in the SW50.1 rotor. The band of purified virus was removed from the gradient, diluted with SSC and pelleted for 1 h at 115 000 g. Two additional cycles of sucrose and CsCl gradient centrifugation were used when analysing viral proteins.

Electron microscopy. Purified virus was resuspended in distilled water and a drop placed onto a Formvar (Electron Microscopy Services, Fort Washington, Pa., U.S.A.) coated grid. A drop of unbuffered 2% phosphotungstic acid (adjusted with NaOH to pH 7) was added for 1 min, the excess removed with a filter paper wedge and the virions were examined with a Philips 300 electron microscope.

Density of virions. The density of [3H]uridine-labelled virus was determined by centrifugation in CsCl gradients. Purified virus was applied to the top of a three-step gradient (see above) which was centrifuged for 24 h in an SW50.1 rotor at 110 000 g at 4 °C. Fractions were collected with a probe and peristaltic pump (Buchler) and pelleted by centrifugation for 1 h at 115 000 g in an SW50.1 rotor (Beckman). The pel of partially purified virus was resuspended in 0·5 ml of SSC, layered onto a three-step CsCl gradient (2·0 ml 40%, 1·5 ml 30% and 1·0 ml 20% CsCl in SSC) and centrifuged for 16 h at 115 000 g in the SW50.1 rotor. The band of purified virus was removed from the gradient, diluted with SSC and pelleted for 1 h at 115 000 g. Two additional cycles of sucrose and CsCl gradient centrifugation were used when analysing viral proteins.

Polyacrylamide gel electrophoresis. To determine the number and mol. wt. of genome RNA segments we used 9% polyacrylamide gels (Laemmli, 1970). The dsRNA was extracted from purified CSV, GSV, CRV, 13p2 and reovirus type 3 (Dearing strain) using an equal volume of phenol saturated with SSC. After mixing for 5 min, the homogenate was centrifuged at 2000 g for 10 min, the aqueous phase removed and the extraction repeated twice. The RNA was precipitated with cold ethanol, collected by centrifugation at 10 000 g for 15 min, suspended in sample buffer and applied to the gel. After electrophoresis for 20 h at 9 mA, the RNA segments were stained with silver (Merril et al., 1981). A semilogarithmic plot of mol. wt. versus relative mobility was constructed using published values for the dsRNA of reovirus type 3 (Ramig et al., 1977). The mol. wt. of each genome segment of CSV, GSV, CRV and 13p2 was estimated by comparing its relative mobility with the plot of dsRNA from reovirus type 3 run in the same gel. The amount of RNA applied to the gel and electrophoresis conditions were varied in some experiments to separate certain bands.

The major virion proteins were analysed using 12.5% polyacrylamide gels. Purified virus was heated (100 °C, 2 min) in sample buffer and applied to the gel. After electrophoresis for 16 h at 4 mA, the gels were fixed and stained with Coomassie Brilliant Blue (Bio-Rad). The following protein mol. wt. standards (Bio-Rad) were run in the
same gel: myosin, 200 000; β-galactosidase, 116 250; phosphorylase B, 92 500; bovine serum albumin, 66 200;
ovalbumin, 45 000; carbonic anhydrase, 31 000; and soybean trypsin inhibitor, 21 500.

RESULTS

Electron microscopy

The four viruses were morphologically identical (Fig. 1). In negatively stained preparations, we observed spherical particles, approximately 75 nm in diameter, with a double capsid shell. Particles viewed along the fivefold axis of symmetry exhibited 20 capsomeres around the periphery of the outer capsid shell. Both complete and incomplete (coreless) particles were seen in most preparations. Density gradient centrifugation commonly yielded two viral bands. The upper band (top component) was largely composed of empty, double-shelled, capsids (Fig. 1) while the lower band contained complete virions. The morphology of the particles was the same as described for mammalian reoviruses (Joklik, 1983b).

Density of the virions

The four viruses had similar densities in CsCl. At equilibrium, two viral bands were seen in each of the gradients. The top component contained incomplete particles with little infectivity or radioactivity. The maximum infectivity and radioactivity both occurred in fractions having densities between 1.34 to 1.36 g/ml. The four isolates had buoyant densities in CsCl similar to the values reported for reoviruses (Joklik, 1983b).

 Electrophoresis of RNA segments

Electrophoresis using SDS–polyacrylamide vertical slab gels revealed that each of the four reoviruses from aquatic animals had a genome composed of 11 segments of double-stranded RNA (Fig. 2). The semilogarithmic plot of mol. wt. versus the migration distance for the reovirus type 3 marker was linear and was used to calculate the weights of the genome segments of the four viruses. The segments fell into three size classes that ranged from $2.5 \times 10^6$ to $0.31 \times 10^6$ mol. wt. (Table 1). There were three large, three medium and five small segments in each genome and each virus had a unique electropherotype. The total genome of each of the viruses was approximately $15 \times 10^6$ mol. wt., consistent with values for other reoviruses (Matthews, 1982). The large segments L1 and L2 of CSV and CRV, L2 and L3 of 13p2 and the small segments S4 and S5 of CRV required extended electrophoresis to separate them. The results obtained differ slightly from the reported mol. wt. of the genome segments of CSV (Winton et al., 1983) and CRV (Hedrick et al., 1984). These differences may be due to variations in the electrophoretic conditions and in the mol. wt. values for reovirus 3 used to determine the size of the segments.

The electropherotypes of the dsRNA from the four viruses were similar; however, the patterns of the viruses replicating optimally in warm water fish cell lines or at higher temperatures (GSV and CRV) were more alike while the viruses with lower temperature optima (CSV and 13p2) seemed to form a second type. Also, the CSV and 13p2 genomes had a higher total mol. wt. than the genomes of CRV and GSV (Table 1). The electrophoretic patterns generated by the RNA segments from the four viruses were unlike those reported for members of the six genera currently defined within the Reoviridae.

Electrophoresis of proteins

Vertical slab gels containing at least 12% polyacrylamide were required to achieve adequate separation of the major virion proteins (Fig. 3). The isolates had two large, one medium and two small virion proteins ranging from 137000 to 30000 mol. wt. (Table 2). Although each virus had a slightly different electrophoretic profile, the relative concentration of the structural proteins was similar for the four isolates. Proteins of approximately 70000 and 34000 mol. wt. were present in the highest concentrations. Polypeptides with these molecular weights are the major outer capsid proteins of mammalian reoviruses (Joklik, 1981). The two large proteins required extended electrophoresis (16 h, 10 mA) to achieve adequate separation, particularly for CRV.
Fig. 1. Electron micrographs of negatively stained virions of the four reoviruses isolated from fish and shellfish, reovirus serotype 3, and an example of top component consisting of empty capsids: (a) GSV, (b) 13p₂, (c) CSV, (d) CRV, (e) reovirus type 3 and (f) top component of CSV. Bar marker represents 100 nm. Virions are approximately 75 nm in diameter.
Fig. 2. Comparative genome profiles of the four reoviruses isolated from fish and shellfish and reovirus serotype 3 (Dearing). Genomic dsRNA was extracted and the gel prepared and stained as in Methods. Lane 1, 13p2; lane 2, CSV; lane 3, GSV; lane 4, CRV; lane 5, reovirus serotype 3. The largest segment at the top of the gel is approximately $2.5 \times 10^6$ mol. wt. while the smallest segment at the bottom is $0.31 \times 10^6$ mol. wt.

We were able to detect several additional minor capsid proteins in some gels but have not been able to compare them sufficiently.

Replication in selected cell lines

The comparative ability of the four viruses to replicate and to produce c.p.e. in fish cell lines was assessed by repeated transfers in FHM, BF-2, CHSE-214, CHH-1 and BB cells. The viruses
Comparison of four aquatic reoviruses

Fig. 3. Comparative structural protein analysis of the four reoviruses isolated from fish and shellfish. The viruses were purified and SDS-PAGE gels prepared and run as in Methods. The gel was stained with 1% Coomassie Brilliant Blue to reveal the five major structural proteins that make up the complete virion of each of the viruses. Mol. wt. of the polypeptides were estimated by comparing the mobilities of the virion proteins with the mobilities of standards (see Methods). Lane 1, 13p2; lane 2, CSV; lane 3, GSV; lane 4, CRV. The largest virion protein was 137,000 and the smallest was 32,000 mol. wt.

replicated well in the cell lines from which they had been originally isolated, but none of the viruses replicated in all lines (Table 3). In some instances, typical c.p.e. was observed on first passage in a particular line but decreased on repeated passage. The two viruses isolated from fish that normally reside in warmer waters, GSV and CRV, grew better in cells having a higher optimal temperature. The CSV and 13p2 isolates grew best in cell lines with lower temperature optima. A common characteristic of these viruses was their capacity to produce plaque-like syncytia in monolayer cultures. The extent of cell fusion was greatly modified by the cell density; at higher densities much smaller syncytial plaques were produced.

Plaque formation

The ability of the viruses to form plaques on selected cell lines is shown in Fig. 4. The viruses produced characteristic small plaques less than 2 mm in diameter. The plaques began as minute
Table 1. Estimates of mol. wt. of RNA segments of four reoviruses from aquatic animals compared to reovirus type 3

<table>
<thead>
<tr>
<th>Segment</th>
<th>13p2</th>
<th>CSV</th>
<th>GSV</th>
<th>CRV</th>
<th>Reo 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>2.40</td>
<td>2.40</td>
<td>2.50</td>
<td>2.40</td>
<td>2.50</td>
</tr>
<tr>
<td>L2</td>
<td>2.30</td>
<td>2.40</td>
<td>2.40</td>
<td>2.40</td>
<td>2.40</td>
</tr>
<tr>
<td>L3</td>
<td>2.30</td>
<td>2.20</td>
<td>2.30</td>
<td>2.30</td>
<td>2.30</td>
</tr>
<tr>
<td>M1</td>
<td>1.90</td>
<td>1.80</td>
<td>1.60</td>
<td>1.60</td>
<td>1.60</td>
</tr>
<tr>
<td>M2</td>
<td>1.70</td>
<td>1.70</td>
<td>1.50</td>
<td>1.50</td>
<td>1.60</td>
</tr>
<tr>
<td>M3</td>
<td>1.40</td>
<td>1.50</td>
<td>1.40</td>
<td>1.30</td>
<td>1.40</td>
</tr>
<tr>
<td>S1</td>
<td>0.85</td>
<td>0.88</td>
<td>0.90</td>
<td>0.90</td>
<td>0.92</td>
</tr>
<tr>
<td>S2</td>
<td>1.80</td>
<td>0.85</td>
<td>0.80</td>
<td>0.88</td>
<td>0.76</td>
</tr>
<tr>
<td>S3</td>
<td>0.57</td>
<td>0.61</td>
<td>0.66</td>
<td>0.61</td>
<td>0.64</td>
</tr>
<tr>
<td>S4</td>
<td>0.47</td>
<td>0.47</td>
<td>0.40</td>
<td>0.41</td>
<td>0.61</td>
</tr>
<tr>
<td>S5</td>
<td>0.35</td>
<td>0.31</td>
<td>0.35</td>
<td>0.40</td>
<td>None</td>
</tr>
<tr>
<td>Total</td>
<td>15.04</td>
<td>15.22</td>
<td>14.71</td>
<td>14.70</td>
<td>14.93</td>
</tr>
</tbody>
</table>

* From Ramig et al. (1977).

Table 2. Estimates of mol. wt. for the five major structural virion proteins of four reoviruses from aquatic animals

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>13p2</th>
<th>CSV</th>
<th>GSV</th>
<th>CRV</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>135</td>
<td>137</td>
<td>137</td>
<td>132</td>
</tr>
<tr>
<td>α2</td>
<td>128</td>
<td>126</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>σ1</td>
<td>70</td>
<td>72</td>
<td>63</td>
<td>70</td>
</tr>
<tr>
<td>σ2</td>
<td>45</td>
<td>44</td>
<td>44</td>
<td>43</td>
</tr>
<tr>
<td>α2</td>
<td>33</td>
<td>34</td>
<td>36</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 3. Cytopathic effect and viral replication of four reoviruses isolated from aquatic animals

<table>
<thead>
<tr>
<th>Virus</th>
<th>Optimum growth temperature (°C)</th>
<th>Original isolation</th>
<th>Cell line</th>
<th>FHM</th>
<th>BF-2</th>
<th>CHSE-214</th>
<th>CHH-1</th>
<th>BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>13p2</td>
<td>15–23</td>
<td>BF-2</td>
<td>-</td>
<td>+ †</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CSV</td>
<td>15–20</td>
<td>CHSE-214</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CRV</td>
<td>25–28</td>
<td>CCO</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GSV</td>
<td>28–30</td>
<td>FHM</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Little or no c.p.e. or viral replication.
† Clear c.p.e. involving the entire monolayer and evidence of viral replication.

Syncytial areas that either became detached from the surface or pulled back toward the plaque edge, often giving a varied plaque type. This difference in plaque appearance was most noticeable in CHSE-214 cells infected with the 13p2 virus (Fig. 4). Some of the plaques that lost the syncytial cells were clear, and others that retained fused cells appeared with dark rings near the periphery or to one side. Counting the plaques was aided by use of a microfilm reader. Most virus–cell combinations produced countable plaques in 5 to 6 days.

The size of plaques produced by each virus varied with the cell type. The golden shiner virus did not produce plaques on CCO cells, barely detectable minute plaques on CHH-1 cells, minute plaques on CHSE-214 cells and countable plaques on FHM cells (not shown). The 13p2 virus produced minute plaques on CCO cells, larger countable plaques on CHSE-214 cells and visible, but fewer plaques on CHH-1 cells. The chum salmon virus produced small plaques on CCO cells, medium plaques on CHH-1 cells and the largest plaques on CHSE-214 cells. Continued incubation of the infected cultures to 8 days did not increase the numbers of plaques.
Comparison of four aquatic reoviruses

Fig. 4. Plaque production of the four reoviruses on CCO, CHH-1 and CHSE-214 cells showing the relatively small plaques characteristic of these viruses. Differences were observed in the ability of each virus to form plaques and in the nature of the plaques produced in monolayers of the various cell lines.

DISCUSSION

The family Reoviridae is composed of viruses with icosahedral morphology whose genomes contain 10 to 12 segments of dsRNA (Matthews, 1982). Particle structure, number of genome segments, host range, and sensitivity to physical and chemical treatments have been used to separate the Reoviridae into six genera (Joklik, 1983a). The four viruses compared in this study had important characteristics in common but could be differentiated from each other and from other members of the Reoviridae. Each of the viruses was a 75 nm spherical particle containing a double capsid layer and a genome composed of 11 segments of dsRNA with a total mol. wt. of approximately 15 × 10^6. These features of morphology and genome organization suggested that the isolates should be regarded as members of the Reoviridae although they did not appear to belong to any of the established genera.

Morphologically, these viruses with their clearly defined double capsid and 20 peripheral capsomeres were most like the members of the genus Reovirus. This structural similarity was also
supported by SDS–PAGE analysis which revealed that virion proteins of 70,000 and 34,000 mol. wt. were present at the highest concentration. Proteins of the same size make up over 60% of the orthoreovirus virion (Joklik, 1981). Another point of similarity was the presence of three size classes of dsRNA. Like members of the genus Reovirus, the isolates had genomes with small, medium and large segments of RNA. However, the description of that genus currently includes only viruses with 10 segments of dsRNA and the members are usually regarded as having homoiothermic vertebrate hosts (Joklik, 1983b).

Reoviruses of the three mammalian serotypes are able to cause haemagglutination of human and other erythrocytes (Joklik, 1983b). Two of the viruses used in this study (CSV and 13p2) have been tested for haemagglutinattion of human type O blood cells and found negative (Winton et al., 1981; Meyers, 1979). Serological tests showed that the CSV and 13p2 viruses are not antigenically related to orthoreoviruses as infectivity of CSV was not neutralized by antisera against the three mammalian reovirus serotypes (Winton, 1981) and the 13p2 virus was not neutralized by antisera against feline reovirus type 3, canine reovirus type 1 or chicken FDO reovirus (Meyers, 1979).

The genus Rotavirus is composed of viruses with 11 segments of dsRNA, but electrophoresis of RNA segments from the five currently recognized groups (Pedley et al., 1986) yields segment patterns quite different from the electropherotypes of these viruses of fish and shellfish. Also, the sharp margin of the outer capsid layer makes the Rotavirus virion quite distinct by electron microscopy (Holmes, 1983). Dot blot hybridization at the 50% stringency level between a 32P-labelled CSV RNA probe and unlabelled group A rotavirus RNA revealed no detectable homology (M. A. McCrae, personal communication). Like reoviruses, the rotaviruses are usually recovered from homoiotherm vertebrate hosts.

The viruses compared in this study seem very different from the other genera of the Reoviridae. Members of the genus Orbivirus have 10 genome segments of dsRNA and a characteristic morphology (Gorman et al., 1983). They are also sensitive to physical treatments such as low pH and high salinity (Palmer et al., 1977), to which these aquatic reoviruses are stable. Cytoplasmic polyhedrosis viruses (CPVs) have 10 segments of dsRNA, are smaller in size, and have a distinctive morphology similar to reovirus cores with 12 spikes or projections at the capsid vertices. CPVs seem to be restricted to arthropod hosts (Payne & Mertens, 1983).

Among the two genera of plant reoviruses, the fijiviruses have 10 segments of dsRNA and the phytoreoviruses 12 (Francki & Boccardo, 1983). In addition to having plant and insect hosts, members of the genera Fijivirus and Phytoreovirus have electropherotypes different from each other and from the viruses examined in this study.

Joklik (1983a) lists CSV and 13p2 as apparent members of the Reoviridae that do not fit within any of the six established genera. Whether the four viruses compared in this study represent a new genus of the Reoviridae or whether they are atypical orthoreoviruses or rotaviruses will not be clear without cross-hybridization studies.

While the four viruses seemed to be members of a common group, the isolates could be distinguished from each other on the basis of specific differences in the mobilities of their RNA segments. These were similar to the variations seen among orthoreoviruses (Fields, 1982), rotaviruses (Pedley et al., 1986) or orbiviruses (Gorman et al., 1981). The viruses could also be separated on the basis of differences in mol. wt. of the virion polypeptides, cell line specificity and optimal temperature. Cross-neutralization studies (Hedrick et al., 1984) showed that three of the viruses (CSV, GSV and CRV) were serologically distinct although slight cross-reactivity was noted, suggesting the three isolates shared one or more antigens.

The four viruses appear to be unique; however, based upon optimum growth temperature, cell line specificity, RNA segment patterns and serological reactions, the isolates from warm water fish (GSV and CRV) were more alike while the viruses with lower temperature optima (CSV and 13p2) appeared similar. The viruses all have a characteristic effect on cells, producing small plaques and well-defined syncytia of fused cells in the monolayers.

To date, all the members of this group have been isolated from aquatic animals although the principal hosts are not known with certainty. A species of fish may be the actual host for the 13p2 virus and its isolation from oysters a result of the efficient filter-feeding mechanisms of these
bivalves (Meyers, 1980). Except for the 13p2 virus, the pathogenicity of these viruses for fish is relatively low. The pathology caused by CSV and 13p2 consisted of focal necrotic lesions in internal organs (Winton, 1981; Meyers, 1980, 1983).

Two other viruses have been reported which are possible members of this group. Nagabayashi & Mori (1983) isolated a virus from oysters (Crassostrea gigas) in Japan that seems to share many of the properties of CSV although no serological or electrophoretic data were reported. On the Chinese mainland, a virus has been discovered that is a serious pathogen of grass carp (Ctenopharyngodon idella) and a probable member of the Reoviridae (Chen & Jiang, 1984). Serological and biochemical analyses are required to determine whether these isolates are related to the four viruses used in this study. It is likely that additional, similar, viruses will be isolated from aquatic animals.

We would like to thank Dr M. A. McCrae for his helpful suggestions. This work is a result of research sponsored by NOAA Office of Sea Grant, U.S. Department of Commerce, under Grant No. NA81AA-D-00086 and the National Science Foundation, U.S.-Japan Cooperative Science Program Grant No. INT-8210495. Oregon State University, Agricultural Experiment Station Technical Paper No. 7895.

REFERENCES


(Received 27 May 1986)