Fish Viruses: Buffers and Methods for Plaquing Eight Agents Under Normal Atmosphere

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A universal procedure was sought for plaque assay of eight fish viruses (bluegill myxovirus, channel catfish virus, eel virus, Egtved virus, infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, lymphocystis virus, and the agent of spring viremia of carp (Rhabdovirus carpio), in dish cultures of various fish cells. Eagle minimal essential medium with sodium bicarbonate-CO$_2$ buffer (Earle’s salt solution) was compared with minimal essential medium buffered principally with tris (hydroxymethyl)aminomethane or N-2-hydroxyethylpiperazine-N’-2’-ethanesulfonic acid at a pH in the range of 7.6 to 8.0 depending upon temperature. Five fish cell lines collectively capable of replicating all fish viruses thus far isolated were tested and quantitatively found to grow comparably well in the three media. Two-phase (gel-liquid) media incorporating the various buffer systems allowed plaquing at 15 to 33 C either in partial pressures of CO$_2$ or in normal atmosphere, but greater efficiency and sensitivity were obtained with the organic buffers, and, overall, the best results were obtained with tris(hydroxymethyl)aminomethane. Epizootiological data, specific fish cell line response, and plaque morphology permit presumptive identification of most of the agents. At proper pH, use of organic buffers obviates the need for CO$_2$ incubators.

Fish virology is relatively youthful; the first isolation was reported in 1960 (13). Since then its growth has been modest, but some aspects have lagged. No scheme has been developed for rapid isolation, differentiation, and identification of fish viruses thus far isolated, particularly those from salmonids. Application of newer techniques similarly has lagged behind virology of other animals.

To date, isolation of eight different fish viruses has been reported. A minimum of four established fish cell lines is required for favorable replication and quantification of these agents and near-optimum temperatures extend from 15 to 30 C. Plaque assay of virus is generally recognized as the most sensitive and accurate method of quantification. Goodheart (4) stated that the plaque method should be used whenever possible. End-point dilution titration has been the standard method of quantifying the fish viruses, although more recently there have been reports in which various procedures for plaque assay were effectively used. Some workers used media with sodium bicarbonate-CO$_2$ buffering in closed vessels (1, 8, 14). Others have used dish cultures of cells under partial pressures of CO$_2$ (6, 9, 10) or under normal atmosphere employing organic buffers (2, 10). In each of these publications the authors deal with only one or two viruses.

We sought a simple method whereby the necessary fish cell lines could be grown in dish cultures under normal atmosphere and used for plaque assay of the fish viruses thus far isolated. Medium L-15 seemed promising, but the brown bullhead line (CCL-59), a critically needed fish cell line, could not be adapted to grow in it. Eagle minimal essential medium (MEM) with fetal bovine serum supported good growth of the necessary cell lines. Therefore, we tested inorganic and organic buffer systems and found the latter to be very promising (11). This report describes a procedure, including a medium, which supports both cell growth and viral replication in normal atmosphere of the eight different fish viruses thus far reported.
The methods implement concurrent isolation and presumptive identification using plaque characteristics.

**MATERIALS AND METHODS**

**Viruses.** Three of the virus strains used in this study are in the American Type Culture Collection, Rockville, Md.: VR 299 strain infectious pancreatic necrosis virus (IPNV), VR 342 strain lymphocystis virus (LV), and VR 686 strain channel catfish virus (CCV). Strain 14, Egved virus, was originally obtained from M. H. Jensen, State Veterinary Serum Laboratory, Aarhus, Denmark. Infectious hematopoietic necrosis virus (IHNV) was obtained from D. Amend, Western Fish Disease Laboratory, Seattle, Wash. Spring virus of carp (*Rhabdovirus carpio*) was obtained from N. Fijan, Veterinary Faculty, Univ. of Zagreb, Yugoslavia. A myxovirus-like agent (5) was isolated by us, as was a small polyhedral cytoplasmic virus of eels (EV-1). We isolated the latter (12) from eels (*Anguilla anguilla*) provided by H. Mann, Bundesforschung Anstalt für Fischerei, Hamburg, Germany. IPNV, CCV, Egved virus, and IHNV used for this work were plaque purified.

**Cells.** We used the following certified cell lines, from the American Type Culture Collection: CCL-42 (FHM, fathead minnow), CCL-55 (RTG-2, rainbow trout gonad), and CCL-59 (BB, brown bullhead).

Low-passage cultures of the BF-2 line (CCL-91), and an uncharacterized line that we started from Micropterus salmoides, the largemouth bass (LBF-1), were used because they were susceptible to both LV and bluegill myxovirus (BMV).

**Culture medium.** Eagle MEM with Earle’s balanced salt (EBSS) unaltered, or with modifications of buffer systems thereto, was used throughout this work. The medium contained final concentrations of 100 International Units of penicillin, 100 μg of streptomycin, and 25 International Units of nystatin per ml. A single lot of fetal bovine serum was used; we added a 10% level for cell growth (MEM-10) but a 2% level was satisfactory and more economical for plaquing (MEM-2).

**Buffer systems.** The standard buffering in MEM-10 was the bicarbonate-CO₂ system provided by EBSS (26 mM NaHCO₃ under partial pressure of CO₂). Modified buffer systems employed 8.9 mM NaHCO₃ plus either 16 mM tris(hydroxymethyl)-ammonomethane(tris)-hydrochloride (Sigma Chemical Co., St. Louis, Mo.) or 14 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES); (Calbiochem, Los Angeles, Calif.). Stock solutions of organic buffers, 1 M Tris or 280 mM HEPES, were prepared to give pH 7.8 at 25°C and were convenient concentrations for use.

**Effect of buffers on cell growth.** Seed cells were grown in static Blake bottle cultures by using MEM-10 with EBSS. Cell sheets were dispersed by a 5- to 10-min exposure to 0.1% trypsin in 1:5,000 ethylenediaminetetraacetic acid (EDTA), suspended in variously buffered MEM-10 at uniform predetermined densities, planted in 60-mm plastic culture dishes (Falcon Plastics, Los Angeles, Calif.), and then incubated at temperatures appropriate for the particular cells. Culture dishes were kept in covered (but not air-tight), semi-rigid plastic freezer trays.

Other than evaporation from the medium, humidity was not provided. When cultures were nearly confluent but still active mitotically, duplicate dishes were taken at random, the populations were dispersed, and samples were counted in a hemacytometer. The mean number of cells in four entire fields, each 0.9 mm², was determined. Two such trials were run for each cell line. As a basis for determining the effect of buffer on cell growth, MEM-10-EBSS was considered to provide standard growth (100%); growth in MEM-10-Tris or MEM-10-HEPES was calculated and expressed as a percentage of the standard.

**Plaque assay.** All virus was plaqueed with a modification (10) of the two-phase (gel-liquid) procedure originally described by Moss and Gravell (9). Buffers were used at the same concentration used for growing cells. Conditions of incubation were those described above. Each virus dilution was plated in duplicate.

**Effect of buffers on plaquing efficiency.** A single lot of cell cultures was used for each trial. Except for the buffer components, the same lot of medium was used for each trial. The same set of virus dilutions, each dilution with its own pipette, was used for each trial. Each trial was run in duplicate or triplicate to provide complete sets of plates of the variously buffered media at two or more durations of incubation; this provided a way of determining the length of time each virus should be incubated for development of favorable plaque size and number. Plaquing of each virus was additionally replicated in two separate trials.

Like the determinations for buffer effect on cell growth, plaque number at a suitable virus dilution was considered to be standard in MEM-2-EBSS, and it was assigned a value of 100%. Plaque numbers in MEM-2-Tris or MEM-2-HEPES were counted and expressed as percentages of the standard.

**Temperature and cell metabolism effects on culture pH.** Most fish viruses replicate through a considerable range of temperature and of pH. Egved and IHNV viruses have temperature optima near 15°C and replicate well at pH 7.6 to 8.0, but not at near-neutral pH. Preliminary work showed that medium buffered at 7.8 to 8.0 worked well, but it was important to determine the effect of different temperatures and of cell metabolism in actual use. Measurements were made of pH of the media buffered with NaHCO₃, Tris, or HEPES which had equilibrated with normal atmosphere at 10, 20, 30°C. We also measured the pH of representative cell cultures after they had grown to near confluency in each of the three media.

**RESULTS**

**Temperature and cell metabolism effects on pH of culture medium.** As could be expected, there was an inverse relationship between pH and temperature in medium with the organic buffers; decrease in temperature...
resulted in a rise in pH, but MEM with HEPES showed less change than when Tris was used (Table 1).

Cell metabolism effected a slight decrease in culture pH, but in CCL-42 and CCL-59, the lines most commonly employed, the change did not go below the level which was favorable for the two most critically sensitive viruses (Table 2).

**Effect of buffers on cell growth.** When compared with cultures grown in MEM-10-EBSS, the established cell lines were found to have grown as well in MEM-10-Tris and MEM-10-HEPES (Table 3). There was 15 to 28% less growth of LBF-1 cells under Tris or HEPES buffers, but, of the two, the results under Tris came closer to the standard.

**Effect of buffers on plaquing efficiency.** All eight viruses produced satisfactory plaques with the three different buffer systems. In general, the use of HEPES and Tris buffers resulted in a significantly greater number of plaques than was obtained when only sodium bicarbonate was used (Table 4). The sole exception occurred with IPNV which had a mean value in HEPES about 7% less than that of the bicarbonate standard. Differences between results with the three buffers were minimal with IPNV and BMV, and they were probably not significant. On the other hand, the response of cells to the other viruses showed an increase of 1.2 to 4.1 times the number of plaques that were obtained with sodium bicarbonate buffer. In addition, the use of organic buffers enhanced the size and number of eel virus plaques; compared to the standard, they were twice as large in HEPES and about four times as large in Tris. Organic buffers quadrupled numbers of EV-1 plaques.

Lymphocystis virus grows slowly, and its "plaques" consist of enormously hypertrophied lymphocystis cells. Such cells were readily distinguished after a week's incubation, but the cells were more easily recognized after 10 or 15 days—about half the time needed for a single-step growth curve. Accordingly, second generation virus was not available for infection. Lymphocystis cells were most easily identified under Tris. All plaques were satisfactorily stained with crystal violet and were readily enumerated after culture sheets were dried. Better visualization of lymphocystis cells was achieved after the stained sheets were cleared either with mineral oil or immersion oil. The best viewing of lymphocystis cells was achieved after differential staining with May-Grünwald-Giemsa.

**Characteristics of fish virus plaques.** Plaques of six of the eight viruses showed distinctive features which were consistent enough in their occurrence to provide presumptive identification (Table 5, Fig. 1). CCV, LV, and BMV each produced cytopathological changes which were unique among the fish viruses. When CCL-55 cells were used, IPNV,

| TABLE 1. Temperature and pH of MEM with organic buffers |
|---------------------------------------------------------|
| Buffer              | Mean pH at equilibration in the absence of cells |
|---------------------|--------------------------------------------------|
|                     | 30°C     | 20°C     | 10°C     |
| 14 mM HEPES + 8.9 mM NaHCO₃ |
| MEM + 10% serum    | 7.90     | 8.09     |          |
| MEM + 2% serum     | 7.95     | 8.10     | 8.16     |
| 2X MEM + 4% serum  | 7.76     | 7.92     | 7.95     |
| 16 mM Tris + 8.9 mM NaHCO₃ |
| MEM + 10% serum    | 8.01     | 8.16     | 8.32     |
| MEM + 2% serum     | 8.02     | 8.27     | 8.36     |
| 2X MEM + 4% serum  | 8.02     | 8.23     | 8.34     |

| TABLE 2. Tabulation of pH values of variously buffered MEM after growth of representative cell lines |
|------------------------------------------------------|
| Cell line    | Cell no. | Temp (C) | Incubation time (days) | Buffer |
|--------------|----------|----------|-----------------------|--------|
| CCL 42*      | 2.5 x 10⁴ | 20       | 3                     | NaHCO₃  | Tris | HEPES |
| (FHM)        | 2.5 x 10⁴ | 25       | 3                     | 7.95    | 8.19 | 8.09  |
|              | 2.5 x 10⁴ | 25       | 3                     | 8.11    | 7.83 | 7.69  |
| CCL 55       | 10⁴      | 20       | 3                     | 7.70    | 7.78 | 7.79  |
| (RTG 2)      | 10⁴      | 20       | 4                     | 7.79    | 7.77 | 7.76  |
|              | 10⁴      | 20       | 4                     | 7.97    | 7.97 | 7.86  |
| CCL 59       | 5 x 10⁴  | 30       | 4                     | 8.30    | 7.93 | 7.86  |
| (BB)         | 10⁴      | 27       | 4                     | 7.90    | 7.90 | 7.75  |
| LBF 1        | 7.5 x 10⁴| 30       | 7                     | 7.64    | 7.64 | 7.45  |

* CCL designates a certified cell line of the American Type Culture Collection, Rockville, Md.
TABLE 3. Comparative growth of four fish cell lines in MEM-10 with three different buffers

| Cell line | Initial no. | Temp (C) | Incubation time (days) | Mean growth |
|-----------|-------------|----------|------------------------|-------------|
|           |             |          |                        | NaHCO₃ | Tris | HEPES |
| CCL 42    | 2.5 x 10⁴   | 20 and 25| 3                      | 5.39 x 10⁴ | 5.63 x 10⁴ | 5.53 x 10⁴ |
| (FHM)     |             |          |                        | (100%) | (104%) | (103%) |
| CCL 55    | 1 x 10⁴     | 20       | 3 and 4                | 1.55 x 10⁴ | 1.67 x 10⁴ | 1.77 x 10⁴ |
| (RTG-2)   |             |          |                        | (100%) | (108%) | (144%) |
| CCL 59    | 1 and 2 x 10⁴ | 27 and 30 | 4 and 9               | 3.25 x 10⁴ | 3.18 x 10⁴ | 2.97 x 10⁴ |
| (BB)      |             |          |                        | (100%) | (98%)  | (91%)  |
| LBF 1     | 7.5 x 10⁴   | 30       | 4 and 7                | 1.95 x 10⁴ | 1.65 x 10⁴ | 1.42 x 10⁴ |
|           |             |          |                        | (100%) | (85%)  | (72%)  |

*For comparative purposes, standard growth (100%) was considered to be the mean that was attained in NaHCO₃ buffering in MEM-10 (EBSS). Growth in MEM-10 with each organic buffer was measured and stated as a mean percentage of the standard growth for each cell line.

TABLE 4. Comparative results of plaquing eight fish viruses in MEM using three different buffers

| Virus                          | Host cell | Temp (C) | Mean plaquing response in MEM with several buffers (%) |
|-------------------------------|-----------|----------|--------------------------------------------------------|
|                               |           |          | NaHCO₃ | HEPES | Tris |
| Rhabdovirus carpio            | CCL 42    | 20       | 100*  | 188   | 207  |
| Bluegill myxovirus            | LBF 1     | 25       | 100   | 101   | 114  |
| Channel catfish               | CCL 59    | 30       | 100   | 183   | 154  |
| Eel                           | CCL 42    | 15       | 100   | 415   | 412  |
| Egtdv                         | CCL 55    | 15       | 100   | 203   | 186  |
| Infectious hematopoietic necrosis | CCL 42 and 55 | 15       | 100   | 124   | 171  |
| Infectious pancreatic necrosis | CCL 55    | 15 and 20 | 100  | 93    | 110  |
| Lymphocystis                  | CCL 91    | 25       | 100   | 133   | 174  |

*Mean plaque number was determined for cultures under MEM with sodium bicarbonate-CO₂ buffering. The mean response of cultures under MEM with organic buffering was determined and related to values obtained with bicarbonate-CO₂ buffering.

IHNV, and Egtdv virus effected a consistent plaque pattern which served to identify the causal agent with a high degree of accuracy. When plated on CCL-42 sheets, the EV-1 produced small plaques (about 0.5 to 1 mm) and Rhabdovirus carpio produced larger plaques (about 2 to 3 mm), but both were otherwise nondescript.

**DISCUSSION**

All eight fish viruses plaqued better in medium with bicarbonate plus an organic buffer than in medium with bicarbonate only. However, the organic buffers alone provided poor cell growth response and so were not employed in plaque assay procedures. The combination of organic buffer and bicarbonate provided cell growth rates which in open dish cultures were comparable to those in bicarbonate and partial pressures of CO₂. The need for CO₂ of homeotherm vertebrate cells and some salmonid fish cells has been established (7). The fish cells with which we worked seem to have a similar requirement, and it appeared that their requirement can be met by bicarbonate.

While the combination of bicarbonate and organic buffer functioned well in open dish culture, we do not recommend its use in sealed systems, for pH declines rather quickly, and cells do not prosper. Of the two organic buffers employed, the Tris-bicarbonate gave slightly better overall results; in addition, it is considerably lower in cost than HEPES buffer.

The fact that a combination buffer system functions well for fish cells and is applicable for plaque assay obviates the need for CO₂ incubators. In homeotherm cell-virus procedures, plaque assay is recognized as providing greater convenience, accuracy, and sensitivity than can be obtained with tube end-point titration methods. We assume that the same applies to poikilotherm cell-virus systems, but we have no
Table 5. Characteristics of plaques of various fish viruses

| Virus         | Cell | Description                                                                 |
|---------------|------|-----------------------------------------------------------------------------|
| Rhabdovirus carpio | CCL 42 | Plaques of 2 to 3 mm formed by necrotic change in cell sheet. Not distinctive. |
| BMV           | CCL 91, LBF 1 | Nuclear and cytoplasmic pyknosis resulting in extreme elongation of fibroblast-like cells only. Jackstraw-shaped cells occur along plaque margin. |
| CCV           | CCL 59 | Replication only in ictalurid cells. Pyknosis followed by cell fusion resulting in massive syncytia followed by contraction and necrosis in annular pattern. |
| EV-1          | CCL 42 | Syncytium formation with contraction and necrosis. Plaques about 0.5 to 1 mm. |
| Egtved        | CCL 52 | Cell necrosis results in plaque with well-defined margins and absence of resistant cells. Plaque center filled with discontinuous but uniformly distributed and finely granular debris. |
| IHNV          | CCL 55 | Plaques characteristically show sheet contraction or dense pile-up along sectors or at times the entire margin. Centers generally open or show coarsely granular debris in discontinuous or partially continuous distribution. |
| IPNV          | CCL 55 | Plaques are typically a reticulum of pyknotic cells which retain an elongate shape and pre-inoculation distribution. Interdigitation of living and dead cells make margins diffuse. Residual living cells are commonly found in centers. |
| LV            | LBF 1 | Gross cellular hypertrophy with concomitant nuclear and nucleolar enlargement. Generalized basophilic and intracytoplasmic DNA inclusions. Plaques are single lymphocystis cells. |

*Deoxyribonucleic acid.

Additional implications and applications were indicated by the results. Strain VR 299 IPNV was used as a reference agent for that virus, but other isolates were also used, and two kinds of plaques were produced. VR 299 virus and several other strains formed easily counted plaques by about 48 h at 20 C. Other strains of IPNV produced less cytopathological change, and their plaques developed more slowly. On a provisional basis, VR 299 plaques are considered IPNV type I; the less-destructive strains are considered to produce type II plaques and require at least 72 h to reach an easily counted size.

The plaquing procedures reported here have been used in research and in routine diagnosis for about 3 years. That experience enables us to make recommendations that should be useful to others who wish to work with fish viruses. Summary-type recommendations for host cells, temperatures, and durations of incubation for practical plaquing of eight fish viruses are given in Table 6.

Eagle (3) has reported on 16 organic buffers or buffer combinations for use with mammalian cell cultures. Those which he recommended included HEPES; moreover, he specified that the organic buffers were additions to media that contained bicarbonate. In the pH range that we used, Tris proved most effective in plaquing fish viruses. Eagle does not recommend Tris in that range but suggests HEPES or tricine. Since two different vertebrate classes are involved, we do not think that the data conflict. If price is not excessive, HEPES and tricine might well be suitable for plaquing fish viruses.

Table 6. Suggested host cell lines and culture conditions for plaquing eight different fish viruses

| Virus                     | Fish cell line | Incubation |
|---------------------------|---------------|------------|
|                           |               | Temp (C)   | Duration prior to fixation |
| Rhabdovirus carpio        | CCL 42        | 20 C       | 3 days                   |
| Bluegill myxovirus         | LBF 1,CCL 91  | 25 C       | 3 days                   |
| Channel catfish virus      | CCL 59        | 30 C       | 40 ± 4 h                 |
| Eel virus                  | CCL 42        | 15 C       | 5 days                   |
| Egtved virus               | CCL 55        | 15 C       | 3 days                   |
| Infectious hematopoietic necrosis virus | CCL 42, 55 | 15 C       | 4 days                   |
| Infectious pancreatic necrosis virus | CCL 55, 42 | 20 C       | 2 days                   |
| Plaque type I              | CCL 55, 42    | 2 days     | 3 days                   |
| Plaque type II             | LBF 1         | 25 C       | 10 ± 3 days              |
| Lymphocystis virus         |               |            |                         |

firm data to support that assumption. Plaque assay provided a significant reduction in time required to obtain results. As an example, the recommended time for incubation of IHNV in tube cultures is 14 days at 15 C. The maximum number of IHNV plaques was obtained in 4 or, at most, 5 days at the same temperature. The time required for lymphocystis virus titration by plaque assay is approximately half that needed for assay in susceptible hosts.
Fig. 1. Comparative plaque characteristics of the four fish viruses that produce acute disease and death. A, B, and C, plaques in CCL-55 cells; D, plaques in CCL-59 cells. A, Egtved virus. B, Infectious hematopoietic necrosis virus. C, Infectious pancreatic necrosis virus. D, Channel catfish virus.

ADDENDUM IN PROOF

An additional rhabdovirus from pike fry (R. Boot- sma and C.J.A.H.V. Van Vorstenbosch, Neth. J. Vet. Sci. 98:86-90.) was kindly provided by P. de Kinkelin, and it has been plaqued by the procedures described above.

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