Precision of a Plaque Assay: Eel Virus European- and Infectious Hematopoietic Necrosis Virus-RTG-2 Cell Systems

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In this paper some criteria for the plaque assay of eel virus European (EVE) and infectious hematopoietic necrosis virus (IHNV) in loosely confluent RTG-2 cell monolayers were evaluated.

1. Suitable virus adsorption times for both EVE and IHNV were 60 min.
2. The linear relationship between the number of plaques and the concentration of virus was shown in both EVE- and IHNV-RTG-2 cell systems. This indicated that a single infectious viral particle of EVE or IHNV is sufficient to infect a RTG-2 cell.
3. The coefficient of variation of the distribution of plaque numbers was 0.06 in the EVE-RTG-2 cell system and 0.054 in the IHNV-RTG-2 cell system.
4. The number of plaques increased as the volume of viral inoculum increased, but the efficiency of adsorption decreased with inocula volumes greater than 0.1 ml.
5. Neither the concentration of agar in agar overlay medium (AOM) nor the incubation periods significantly affected the number of plaques (EVE: 0.5<\text{P}, 0.5<\text{P}; IHNV: 0.25<\text{P}, 0.5<\text{P}, respectively). However there was a significant effect of both concentration of agar in AOM and incubation period on the diameter of plaques (EVE: \text{P}<0.05, \text{P}<0.005; IHNV: \text{P}<0.005, \text{P}<0.01, respectively).

These results indicated that loosely confluent RTG-2 cell monolayers gave precise quantitative analysis for EVE and IHNV.

Introduction

WOLF and QUIMBY (1973a, b) were the first to describe a general procedure for the plaque assay of fish viruses. This procedure is routinely used in fish virology laboratories for detection, diagnosis and enumeration of viruses. Among those available the RTG-2 cell line is preferred by many because of its sensitivity and broad range of virus susceptibility (in review of WOLF and MANN, 1980). OKAMOTO et al. (1983b) recently showed that the confluent condition of RTG-2 cells was classified into two phases according to the cell density and viral infectivity titers were highest in loosely confluent cell monolayers. There are no reports of the precision and quantitativeness of the plaque assay when using the RTG-2 cell line.

In order to utilize the plaque assay quantitatively the following criteria should be met; specificity in plaque formation (morphology, size), linearity of the number of plaques with the dilutions of virus and reproducibility in virus inoculum volumes to plaque numbers (KITAMURA, 1976). BURKE and MULCAHY (1980) reported on the precision and quantitativeness of the plaque assay with infectious hematopoietic necrosis virus (IHNV) in the EPC cell line. In this paper the same criteria for the plaque assay of eel virus European (EVE) and IHNV in loosely confluent RTG-2 cell monolayers are evaluated.

Materials and Methods

Cell Line and Virus

RTG-2 cells, EVE and IHNV used in this study were the same as those previously reported (OKAMOTO et al., 1983b). Eel virus European was serologically classified to Group III of infectious pancreatic necrosis virus by OKAMOTO et al. (1983a) and the biochemical and biophysical structures have been described by HEDRICK et al. (1983). The infectious hematopoietic necrosis virus used was isolated from rainbow trout fry in
Plaque Assay

The plaque assay used was a modification of the procedure described by Wolf and Quimby (1973a, b). They used a two-phase overlay (gel-liquid overlay) for the plaque assay of eight fish viruses in normal atmosphere. In this study a single overlay plaque assay was performed for EVE and IHNV. A moist chamber was used for the protection of cell monolayers from damage due to drying. Purified agar (Difco Lab.) was used for the gel phase overlay medium. RTG-2 cells used for the plaque assay were loosely confluent cell monolayers (approximately 6.0 x 10⁴ cells per cm²; Okamoto et al., 1983b). The pH of the medium was adjusted to 7.6-7.8 with 14 mM Hepes buffer. The plastic tissue culture petri dishes (50 mm internal diameter: 60 x 15 petri dishes; Corning Ltd.) were used in each assay. Unless otherwise noted the following conditions were part of each assay. The concentration of agar in agar overlay medium (AOM) was 0.8%. Suitable virus dilutions containing approximately 20-50 plaque forming units (pfu) for EVE and 100-200 pfu for IHNV were prepared with Hanks' balanced salt solution (BSS); pH was adjusted to 7.6-7.8 with 16 mM Tris-HCl or 14 mM Hepes buffers. Virus inoculum was added in 0.15 ml aliquots per dish and plated in triplicate. Virus adsorption time was 60 min for both EVE and IHNV at 15-20°C. During virus adsorption, the inoculum was distributed over the cell sheet by a gentle tilting of the petri dishes at 15 min intervals. After adsorption, virus inoculum was removed using a syringe and 5.0 ml of AOM (37-38°C) was then added to each petri dish. Plaques were allowed to form for 3 days for EVE and 5 days for IHNV at 18°C. One ml of full strength formalin (37% formaldehyde) was added to each dish and cell fixation was complete after 1 hour. The cells were then stained with a solution of 1% crystal violet and the number of plaques and diameter of plaques was determined.

Virus Adsorption Time

Virus adsorption time was examined at 10 min intervals from 10 to 60 min for EVE and from 10 to 120 min at 10 or 20 min intervals for IHNV.

Linear Relationship between the Number of Plaques and the Concentration of Virus Inoculum

Serial 10-fold dilutions of EVE and serial 2-fold dilutions of IHNV were made in Hanks' BSS. Viral titers of each dilution were then measured by the plaque assay mentioned above.

Reproducibility of the Number and Distribution of the Diameter of Plaques

In order to confirm the reproducibility of the number of plaques which formed on loosely confluent RTG-2 cell monolayers, 10 petri dishes were used for both EVE and IHNV. Five of the ten petri dishes used to confirm the reproducibility of the number of plaques were selected at random to determine the distribution of the diameter of plaques of EVE and IHNV. The diameter of plaques was measured on high magnification with an electric magnifying glass (Nikon Co.).

Relationship between the Number of Plaques and the Volume of Virus Inoculum

Virus inoculum was prepared to dilute the stock viruses with Hanks' BSS to 20-200 pfu per aliquot in a volume of 0.1, 0.15, 0.2, 0.4 or 0.6 ml.

Effects of Different Agar Concentrations in Overlay Medium on the Number and Diameter of Plaques

Agar concentrations of 0.8, 1.0 and 1.2% for EVE and 0.65, 0.8, 1.0, and 1.2% for IHNV in the overlay medium were tested. The incubation period for plaque formations was 3 to 5 days for EVE and 5 to 7 days for IHNV at 18°C.

Results

Virus Adsorption Time

Maximum virus adsorption for EVE and IHNV to RTG-2 cell sheets was 40 min and 60 min respectively. Virus adsorption times between 40 and 60 min with 0.15 ml of EVE suspension failed to increase plaque titers (Fig. 1). Suitable virus adsorption times for both EVE and IHNV were 60 min.

Linear Relationship between the Number of Plaques and the Concentration of Virus

The number of plaques that developed on a series of cell cultures infected with serial dilutions of both EVE and IHNV was proportional to the
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**Fig. 1.** Virus adsorption curves of EVE and IHNV. The marks of ● and ○ show EVE and IHNV, respectively.

**Fig. 2.** Linear relationship between the number of plaques and the virus concentration. Ten-fold virus dilutions were assayed for EVE (●) and 2-fold dilutions were assayed for IHNV (○). The number of plaque forming unit (pfu) per 0.15 ml of each virus dilution was determined.

Reproducibility of the Number and Distribution of the Diameter of Plaques

The reproducibility of the assay as measured by the number of plaques in replicated platings is shown in Table 1. The coefficient of variation of the distribution of plaque numbers is an index for precision and values of 0.06 in the EVE-RTG-2 cell system and 0.054 in the IHNV-RTG-2 cell system were obtained. Distribution of the diameter of plaques is shown in Fig. 3. The diameter of plaques formed by EVE was measured on cells fixed 3 days after virus inoculation and the mean ± standard deviation was 2.26 ± 0.80 mm. The diameter of plaques formed by IHNV was measured on cells fixed 5 days after virus inoculation was 0.84 ± 0.30 mm.

Relationship between the Number of Plaques and the Volume of Virus Inoculum

The number of plaques increased as the volume of viral inoculum increased, but the efficiency of adsorption decreased with inocula volumes greater than 0.1 ml. For example, the ratios of the efficiency of adsorption for EVE were 0.76, 0.64, 0.41 and 0.30 for 0.15, 0.2, 0.4 and 0.6 ml volumes.

<table>
<thead>
<tr>
<th>Table 1. Reproducibility of the number of plaques</th>
</tr>
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<tbody>
<tr>
<td>plaque numbers/0.15 ml/dish</td>
</tr>
<tr>
<td>EVE</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>19</td>
</tr>
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<td>23</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>21</td>
</tr>
</tbody>
</table>

n indicates the number of petri dishes. 

<table>
<thead>
<tr>
<th>n</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>20.7</td>
</tr>
<tr>
<td>SD</td>
<td>1.25</td>
</tr>
<tr>
<td>CV</td>
<td>0.060</td>
</tr>
</tbody>
</table>

* n indicates the number of petri dishes.
* x indicates the mean of plaque numbers.
* SD indicates the standard deviation.
* CV is the abbreviation of the coefficient of variation and indicates SD/x.
Fig. 3. Distribution of the diameter of plaques. The diameter of plaques of EVE was measured 3 days after inoculation and that of IHNV was measured 5 days after inoculation. The signs of n, \( \bar{x} \), SD and CV show the total number of plaques, the mean, the standard deviation and the coefficient of variation, respectively.

Table 2. Relationship between the number of plaques and the volume of inoculum for EVE and IHNV

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>EVE</th>
<th>IHNV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental value</td>
<td>Expected*¹ value</td>
</tr>
<tr>
<td>0.1</td>
<td>27.3</td>
<td>27.3</td>
</tr>
<tr>
<td>0.15</td>
<td>31.0</td>
<td>41.0</td>
</tr>
<tr>
<td>0.2</td>
<td>35.0</td>
<td>54.6</td>
</tr>
<tr>
<td>0.4</td>
<td>45.3</td>
<td>109.2</td>
</tr>
<tr>
<td>0.6</td>
<td>49.3</td>
<td>163.8</td>
</tr>
</tbody>
</table>

*¹ Expected value was calculated on the assumption that the adsorbing efficiency of virus was the same as that of 0.1 ml volume.

*² Adsorbing efficiency was shown as the ratios which divided Experimental value by Expected value.
centration in AOM. Neither the concentration of agar in AOM nor the incubation periods significantly affected the number of plaques (EVE: 0.5<P, 0.5<P; IHNV: 0.25<P, 0.5<P, respectively). There was a significant effect of both concentration of agar in the AOM and incubation period on the diameter of plaques (EVE: P<0.05, P<0.005; IHNV: P<0.005, P<0.01, respectively).

Discussion

Suitable virus dilutions may be added to the cells in small volumes (e.g. 0.1-0.5 ml) to encourage rapid adsorption (COOPER, 1967). However, it may under certain conditions be easier to adsorb virus in larger volumes of inoculum. This can be done provided the efficiency of viral adsorption does not decline (KITAMURA, 1976). In this study volumes of inoculum greater than 0.1 ml had corresponding losses in efficiency (Table 2). The 0.1 ml volume of inoculum is therefore minimum volume for virus adsorption without cell damage brought by drying in our experience. But occasionally even at this volume, some cell damage can occur. Since viral adsorption efficiency in the 0.15 ml volume of inoculum was about 80% for EVE and about 70% for IHNV in comparison with that in 0.1 ml, the 0.15 ml volume of inoculum was used in this study for totally eliminating the risk of cell damage by drying. BURKE and MULCAHY (1980) experienced heat damage due to the agar overlay medium in EPC cells. These cells have an upper limit of 33°C for cell growth temperature (FIJAN et al., 1983). The range and optimum of RTG-2 cells growth temperature are 4-26°C and 20°C, respectively (WOLF and QUIMBY, 1962). In our experience, RTG-2 cells tended to suffer from more thermal damage than EPC, FHM and CHSE214 cells with a warm agar overlay. In addition we found RTG-2 required more humidity during virus adsorption than the other cells lines. Therefore, special attention to retain humidity in petri dishes during virus adsorption and to avoid the thermal damage caused by gel phase overlay medium were important steps in the procedure. A damp cloth placed over the covered petri dishes prevented dehydration. Cooling the gel phase overlay medium to 37-38°C and pouring into petri dishes on an aluminum plate on ice minimized thermal damage.

The number of plaques formed from animal viruses on cell monolayers does not always follow the Poisson distribution because cells vary in size, surface properties, etc., but usually the deviations are small enough to be negligible (DULBECCO and GINSBERG, 1973). If the coefficient of variation (the standard deviation divided by the mean) in the distribution of the plaque numbers formed in each of 10-20 plates is less than 0.15, the system can be utilized for quantitative analysis (DOUGHERTY, 1964; GIL-FERNANDEZ et al., 1976; KITAMURA, 1976). When EVE and IHNV were assayed on loosely confluent RTG-2 cell monolayers, the coefficient of variation of the distribution of plaque numbers formed in each plate was 0.06 in the EVE-RTG-2 cell system and 0.054 in the IHNV-RTG-2 cell system. This indicated that loosely confluent RTG-2 cell monolayers gave precise and quantitative analysis for EVE and IHNV. The susceptibility of loosely confluent RTG-2 cell monolayers was nearly constant and very little fluctuation was noticed. Therefore, assuming that virus infectivity of EVE and IHNV is constant, the number of plaques of both viruses on loosely confluent RTG-2 cell monolayers can be applied to the Poisson distribution in the plaque assay. The standard deviation (SD) of the Poisson distribution: SD = √x/n, where x is the mean value of plaque numbers in (n) the number of petri dishes, the coefficient of variation serves as a relative measure of precision: SD/x = 1/√n x. The smaller the coefficient of variation, the greater the precision. The accuracy of the assay is shown when 95% of all observations made fall within two standard deviations from the mean in either direction. Plaque size should not effect the accuracy if plaques per plate give no significant loss by overlapping and if the internal diameter of the petri dish is at least 25 times as large as the average plaque diameter (COOPER, 1967). The allowable maximum density varies with the size of the plaques and the sharpness of their margins (DULBECCO and GINSBERG, 1973). The average plaque diameters of EVE and IHNV are 2.26 mm and 0.48 mm, respectively in the virus-RTG-2 cell system (Fig. 3). Estimating from the average plaque diameter of EVE and
IHNV on the basis of Cooper (1967), the diameter of petri dishes for the virus assays of EVE and IHNV should be at least 57 mm and 16 mm, respectively. The internal diameter of the petri dish used in this study was 50 mm. This size is slightly small for EVE and the suitable plaque numbers without overlapping counted on the plate were under 70 from our experience. On the other hand, the size is adequate for IHNV and the suitable plaque numbers counted on the plate without overlapping were under 200. If it is estimated that the coefficient of variation is 0.1, a total of 100 plaques must be theoretically counted in all of the plates. Under these conditions, the accuracy of the assay is ±20% and its results will fall between 40 and 60 plaques in 95% of the cases. Assuming that suitable virus dilutions are prepared to form 20 and 100 plaques per plate for EVE and IHNV respectively, the number of petri dishes required to keep the accuracy of ±20% is five for EVE and one for IHNV. To keep an accuracy of ±10% under the same assumption as above, fourth as many petri dishes must be prepared for each virus inoculum.

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References


Okamoto, N., T. Shirakura, Y. Nagakura and T. Sano (1983b): The mechanism of interference with fish viral infection in the RTG-2 cell line. Fish Pathology, 18, 7–12.


