魚病研究 Fish Pathology 22 (3) 147–152, 1987. 9

Plaque Assay of *Oncorhynchus masou* Virus (OMV)

Yuto KAMEI, Mamoru YOSHIMIZU, and Takahisa KIMURA

Laboratory of Microbiology, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan

(Received January 21, 1987)

Plaque assays for salmonid virus, *Oncorhynchus masou* virus (OMV), were examined and compared with infectious haematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV), combining various fish cell-lines and three overlays.

OMV produced the plaques only in chinook salmon embryo (CHSE-214) cells and rainbow trout gonad (RTG-2) cells, irrespective of overlays used, but plaque formation of IHNV and IPNV differed in different cell-lines according to the overlays used. The plaquing procedure using CHSE-214 cells gave a ten-fold higher number of plaques than RTG-2 cells.

Among three overlays of methylcellulose, gum tragacanth, and agarose, methylcellulose overlay facilitated OMV plaque formation, and the plaques were most clearcut and easily enumerated. On the whole, the combination of methylcellulose overlay and CHSE-214 cell-line was the most suitable for plaque assay of OMV as well as IHNV and IPNV.

Oncorhynchus masou virus (OMV), salmonid herpesvirus 2, was first isolated from ovarian fluids of landlocked masu salmon (*Oncorhynchus masou*) in 1978 (KIMURA *et al.*, 1981a; ROIZMAN *et al.*, 1981). This virus not only causes hepatic necrosis in salmon fry, but also epidermal tumors for those fry which survived after experimental infection (KIMURA *et al.*, 1981a, b; YOSHIMIZU *et al.*, 1986). The sensitivity of various fish cell lines to infection by OMV as well as infectious haematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) (SCHERRER and COHEN, 1975; KELLY *et al.*, 1978; FENDRICK *et al.*, 1982) has been previously studied (KIMURA *et al.*, 1981a). Although the biochemical and physicochemical properties of OMV have been determined, preferable plaquing procedures have not been reported.

Types of overlays used for viral plaque assay are described as liquid (IBRAHIM and LOH, 1972; BUCH and LOH, 1985), semisolid (MIRCHAMSY and RAPP, 1968; DOBOS, 1976; ROBIN *et al.*, 1982), solid (HOLLAND and MCLAREN, 1959; GABRIELSON and HSIUNG, 1965; CAMPBELL and WOLF, 1969; MACDONALD, 1978), and solid-liquid (Moss and GRAVELL, 1969). DOBOS (1976) reported the use of a gum tragacanth overlay for plaque assay of fish viruses, while BURKE and MULCAHY (1980) determined that a gum tragacanth or methylcellulose overlay was superior to an agarose overlay for the plaque assay of IHNV. This report describes the optimal plaque forming condition for OMV using various combinations of cell lines and overlays, and compares it with the plaquing efficiency of IHNV and IPNV.

Materials and Methods

Viruses

OMV (strain 00-7812) and IHNV (strain ChAb) were isolated in our laboratory from masu salmon (*Oncorhynchus masou*) and chum salmon (*O. keta*) respectively. IPNV (strain VR299) was provided by Dr. R. P. HEDRICK, University of California, Davis. These stock viruses were propagated at 15°C using rainbow trout (*Salmo gardneri*) gonad cells (RTG-2; WOLF and QUIMBY, 1962) in 75 cm^2 tissue culture flasks (Falcon) containing 25 m*l* of MEM10-Tris, composed of Eagle's minimum essential medium (MEM, Gibco), 10% fetal bovine serum (M. A. Bioproduct), 0.075% $NaHCO_3$, 100 IU/m*l* penicillin (Sigma), 100 μg/m*l* streptomycin (Sigma), and 1.6% Tris buffer (Tris (hydroxymethyl) aminomethane (Tris)-hydro-

chloride) (Sigma) adjusted to pH 7.8. When the cytopathic effect was complete, the culture fluid was removed from the flasks and clarified by centrifugation at 4,000 rpm at 4°C for 20 min. The supernatants were filtered through a 0.40 μm pore-sized filter (Nuclepore) for OMV and a 0.45 μm pore-sized filter (Millex-HA, Millipore) for both IHNV and IPNV. The viruses were stored at −80°C until used. The titres of these stocks of OMV, IHNV, and IPNV were 4.05, 5.80, and 8.30 (log $TCID_{50}$/m*l*) respectively.

Cell cultures

Bluegill fry (BF-2; WOLF and QUIMBY, 1966), brown bullhead (BB; WOLF and QUIMBY, 1969), chinook salmon embryo (CHSE-214; FRYER *et al.*, 1965), epithelioma papillosum carpio (EPC; TOMASEC and FIJIAN, 1971), fathead minnow (FHM; GRAVELL and MALSBERGER, 1965), steelhead trout embryo (STE-137; FRYER *et al.*, 1965), and RTG-2 cell lines were used for the comparative plaque assay. Cells were grown in MEM10-Tris at 15°C with the exception of BB cells which were incubated at 20°C. Each well of a 24-well plate (16 mm in diameter, Falcon) was seeded with 1 m*l* of growth medium to give approximately 10^6 cells/m*l*/well. The 1-day-old confluent monolayers of these cells were used for the plaque assay.

Overlay media

Overlays used in the comparative plaque assay were 0.8% methylcellulose (4,000 centipoise, Wako), 0.8% gum tragacanth (Wako), and 0.8% agarose (Wako). The overlay media were prepared with double strength MEM2-Tris which was a slightly modified method of BURKE and MULCAHY (1980).

Plaquing procedures

Serial 10-fold dilutions of viruses were made by using Hanks' balanced salt solution (Hanks' BSS, Gibco) supplemented with 0.0375% $NaHCO_3$, 100 IU/m*l* penicillin, and 100 μg/m*l* streptomycin. Drained cells were inoculated with 0.1 m*l* of each dilution, and allowed virus to adsorb for 1 h at 15°C. Following adsorption, the cells were rinsed twice with 0.5 m*l* Hanks' BSS and 1 m*l* of individual overlay was added to each well. In the case of the agarose overlay, the overlay was added in drops to cells cooled on ice. Plates were incubated at 15°C for 5 days for IHNV and 7 days for OMV and IPNV. Then, the cells were fixed with 10% formalin and stained with 0.1% crystal violet. Plaques were counted and their size was estimated. The numbers and sizes of the plaques were expressed as mean values of three replicates.

Results

Optimal condition for plaque formation of OMV

The plaquing efficiency of OMV was surveyed using seven fish cell-lines and three kinds of overlays, comparing with IHNV and IPNV. Among the seven cell-lines tested, OMV formed the plaques only in CHSE-214 and RTG-2 cells (Table 1). Optimal plaquing efficiency was obtained using a methylcellulose overlay and CHSE-214

Table 1. Effects of three different overlays and seven fish cell-lines on OMV plaquing efficiency

Cell-lines	Average of three trials					
	PFU/m*l*			Plaque size (mm)		
	Methyl-cellulose	Tragacanth	Agarose	Methyl-cellulose	Tragacanth	Agarose
BB	0.0	0.0	0.0			
BF-2	0.0	0.0	0.0			
CHSE-214	7.6×10^3	6.9×10^3	1.6×10^3	0.3–0.6	0.3–0.4	0.8–1.0
EPC	0.0	0.0	0.0			
FHM	0.0	0.0	0.0			
RTG-2	6.5×10^2	4.2×10^2	$1.5\times10^{2*}$	0.9–1.7	0.7–1.2	0.6–1.3
STE-137	0.0	0.0	0.0			

* Plaque count on one trial.

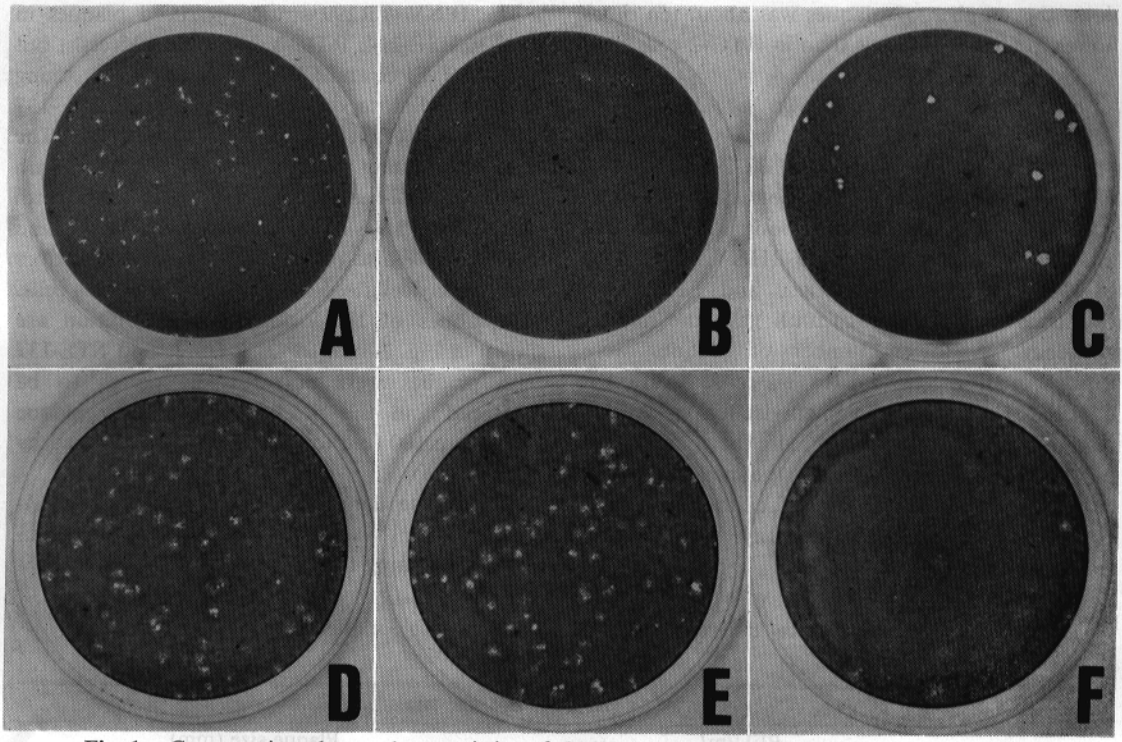

Fig. 1. Comparative plaque characteristics of OMV grown under three overlays. A, methylcellulose overlay on CHSE-214 cells; B, tragacanth overlay on CHSE-214 cells; C, agarose overlay on CHSE-214 cells; D, methylcellulose overlay on RTG-2 cells; E, tragacanth overlay on RTG-2 cells; F, agarose overlay on RTG-2 cells.

Table 2. Effects of three different overlays and seven fish cell-lines on IHNV plaquing efficiency

Cell-lines	Average of three trials					
	PFU/m*l*			Plaque size (mm)		
	Methyl-cellulose	Tragacanth	Agarose	Methyl-cellulose	Tragacanth	Agarose
BB	0.0	0.0	0.0			
BF-2	1.6×10^5	2.4×10^5	5.0×10^4	0.3–0.4	0.4–1.0	0.5–1.6
CHSE-214	1.1×10^6	9.9×10^5	7.9×10^{5}*1	0.4–0.6	0.4–0.7	0.5–1.0
EPC	2.7×10^6	1.7×10^6	ND	0.3–0.6	0.4–0.9	
FHM	2.4×10^6	2.6×10^6	2.9×10^{5}*1	0.4–0.7	0.5–0.9	1.5–3.1
RTG-2	4.0×10^4	3.2×10^4	1.6×10^{4}*2	1.0–1.8	0.4–1.0	0.7–1.8
STE-137	0.0	0.0	0.0			

*1 Average of two trials; *2 plaque count on one trial; ND, not determined.

cells, and in this combination the plaque was clear and easily visible (Fig. 1). OMV produced small indistinct plaques under gum tragacanth which could not be counted precisely during the 7-day incubation period. The agarose overlay produced lower, but larger plaques in comparison with the other two, and results were not reproducible, possibly due to heat damage to the cells. Although OMVproduced almost the same plaque diameter (0.6–1.7 mm) in RTG-2 cells under the three over-

lays, the number of plaques was about ten times lower in CHSE-214 cells than in RTG-2 cells.

Comparative plaque formations of IHNV and IPNV with OMV

IHNV produced plaques in BF-2, CHSE-214, EPC, FHM, and RTG-2 cells, but not in BB and STE-137 cells (Table 2). Among the cells that allowed plaque formation of IHNV, EPC cells overlayed with methylcellulose and FHM cells overlayed with gum tragacanth gave the highest efficiency. A lower number of plaques were formed in BF-2 cells and RTG-2 cells. Although CHSE-214 cells with a methylcellulose overlay resulted in approximately half the number of plaques of EPC or FHM cells, the methylcellulose overlay-CHSE-214 cell system facilitated counting because it produced clear and easily visible plaques as observed for OMV (Fig. 2).

The IPNV VR299 strain produced plaques in BF-2, CHSE-214, RTG-2, and STE-137 cells, but not in BB, EPC, and FHM cells (Table 3). Using a methylcellulose overlay in BF-2 and STE-137 cells, IPNV failed to produce plaques. The highest plaquing efficiency was obtained with CHSE-214 cell-methylcellulose or gum tragacanth overlay ($1.6–1.9\times10^7$ PFU/m*l*). Like OMV and IHNV, IPNV also produced more distinct plaques using methylcellulose overlay and CHSE-214 cells. The plaques of IPNV under this condition are shown in Fig. 2. In BF-2, RTG-2, and STE-137 cells, plaques were not distinct enough to be measured and the titres determined by plaque assay were 100 fold lower than those produced by $TCID_{50}$.

From these data, the methylcellullose overlay-CHSE-214 cell system proved to be the most suitable for plaquing the three fish viruses tested.

Table 3. Effects of three different overlays and seven fish cell-lines on IPNV plaquing efficiency

Cell-lines	Average of three trials					
	PFU/m*l*			Plaque size (mm)		
	Methyl-cellulose	Tragacanth	Agarose	Methyl-cellulose	Tragacanth	Agarose
BB	0.0	0.0	0.0			
BF-2	0.0	2.5×10^6	7.9×10^6		0.5–1.0	1.0–2.8
CHSE-214	1.6×10^7	1.9×10^7	$7.7\times10^{6*1}$	0.4–0.6	1.2–2.2	1.0–3.1
EPC	0.0	0.0	0.0			
FHM	0.0	0.0	0.0			
RTG-2	2.7×10^6	3.5×10^6	$1.7\times10^{5*2}$	0.2–0.6	0.7–1.4	1.2–3.7
STE-137	0.0	4.2×10^6	$8.5\times10^{6*2}$		0.6–1.4	1.1–3.5

*1 Average of two trials; *2 plaque counts on one trial.

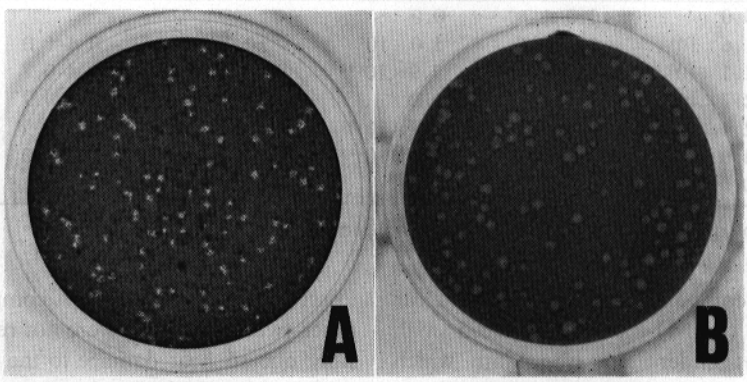

Fig. 2. Plaque characteristics of IHNV and IPNV grown under methylcellulose overlay on CHSE-214 cells. A, IHNV; B, IPNV.

Table 4. Comparison of plaque assay with $TCID_{50}$ method on CHSE-214 cell-line

Viruses	Plaque assay (PFU/ml)*	$TCID_{50}$/ml
IPNV	1.6×10^{7}	1.2×10^{7}
IHNV	1.1×10^{6}	6.3×10^{5}
OMV	7.6×10^{3}	1.2×10^{4}

* Plaque assay was carried out using methylcellulose overlay.

Comparative titres determined by plaque assay and $TCID_{50}$ method

The virus titres determined by plaque assay using methylcellulose and CHSE-214 cells were compared with the $TCID_{50}$ titres in the same cells (Table 4). OMV showed slightly lower titres in the plaque assay compared with the $TCID_{50}$. In contrast to the results of OMV, titre of IHNV in the plaque assay was higher than in the $TCID_{50}$. IPNV displayed similar titres in both the plaque assay and the $TCID_{50}$ method.

Discussion

In this study, the twenty-one combinations of methylcellulose, gum tragacanth, and agarose overlays and seven fish cell-lines were tested to compare the plaque forming efficiency of OMV with the plaque formation of IHNV and IPNV. KLEEMAN *et al.* (1970) reported that salmonid fish cells require CO_2 for their growth *in vitro*. However, EAGLE (1971) demonstrated that an organic buffer or buffer combination is available for reduction of pH variation in the mammalian cell culture. WOLF and QUIMBY (1973) recommended using Tris-buffer for fish cell culture. Thus, we selected a medium supplemented with Tris-buffer in the open air instead of using a CO_2-incubator for cell cultures. The data obtained suggested that adequate plaque formation was obtained under these conditions. Although virus plaque formation is known to be enhanced by the addition of DEAE dextran (ROSSI and WATRACH, 1970) or polybrene (LEONG *et al.*, 1981), use of these additives was omitted in order to simplify the procedure.

In the present study, OMV plaque formation was observed on only two of the tested cells, those being CHSE-214 and RTG-2 cells. The plaque titre was about ten times higher in CHSE-214 cells than in RTG-2 cells. For both of these types of cells, methylcellulose overlay was more effective on OMV plaque formation than others. On the whole, agarose overlay was difficult to handle and often caused cell or virus damage. Therefore, among the three different overlays, methylcellulose was considered to be the best. Furthermore, the other cells, except for CHSE-214 cells, were not suitable for plaque assay of OMV because of low plaque counts, obscure morphology, and difficulty in enumeration.

IHNV produced plaques in BF-2, CHSE-214, EPC, FHM, and RTG-2 cells regardless of the overlays tested, but not in BB and STE-137 cells. Among the cells which formed plaques, FHM cells were most sensitive to IHNV in reference to the plaque counts and less plaques were produced in RTG-2 cells than FHM cells by 10 to 10^2 order. These results were in accordance with those of other studies (KELLEY *et al.*, 1978; FENDRICK *et al.*, 1982). The plaques in CHSE-214 cells, however, facilitated visual enumeration more than the others.

IPNV formed the plaques in BF-2, CHSE-214, RTG-2, and STE-137 cells overlayed with gum tragacanth or agarose. Methylcellulose overlay seemed to inhibit plaquing IPNV in BF-2 and STE-137 cells. Plaques were not observed in FHM cells infected with a higher dilution of IPNV. Since SCHERRER and COHEN (1975) and NICHOLSON *et al.* (1979) have reported that the plaquing of RTG-2 propagated IPNV was restricted considerably in FHM cells, if IPNV is inoculated in a higher dilution into FHM cells, it is likely that FHM cells may not allow IPNV to form plaques. Because the IPNV plaques in CHSE-214 cells were five times as great as the plaques in other cells, CHSE-214 cells were found to be most suitable for plaque assay of IPNV. In summary, methylcellulose overlay and the CHSE-214 cells was the most suitable combination for plaque assay of OMV as well as IHNV and IPNV.

References

BUCH, C. D. and P. C. LOH (1985): Liquid overlay plaquing of channel catfish virus. *J. Fish Diseases*, **8**, 325–328.

BURKE, J. A. and D. MULCAHY (1980): Plaquing procedure for infectious hematopoietic necrosis virus. *Appl. Environ. Microbiol.*, **39**, 872–876.

CAMPBELL, J. B. and K. WOLF (1969): Plaque assay and some characteristics of Egtved virus (virus of viral hemorrhagic septicemia of rainbow trout). *Can. J. Microbiol.*, **15**, 635–637.

DOBOS, P. (1976): Use of gum tragacanth overlay, applied at room temperature, in the plaque assay of fish and other animal viruses. *J. Clin. Microbiol.*, **3**, 373–375.

EAGLE, H. (1971): Buffer combinations for mammalian cell culture. *Science*, **174**, 500–503.

FENDRICK, J. L., W. J. GROBERG, and J. C. LEONG (1982): Comparative sensitivity of five fish cell lines to wild type infectious haematopoietic necrosis virus from two Oregon sources. *J. Fish Diseases*, **5**, 87–95.

FRYER, J. L., A. YUSHA, and K. S. PILCHER (1965): The *in vitro* cultivation of tissue and cells of pacific salmon and steelhead trout. *Ann. N.Y. Acad. Sci.*, **126**, 566–586.

GABRIELSON, M. O. and G. D. HSIUNG (1965): Sensitivity of agar overlay method for the recognition of enteroviruses. *Appl. Microbiol.*, **13**, 967–972.

GRAVELL, M. and R. G. MALSBERGER (1965): A permanent cell line from the fathead minnow (*Pinephales promelas*). *Ann. N.Y. Acad. Sci.*, **126**, 555–565.

HOLLAND, J. J. and L. C. MCLAREN (1959): Improved method for staining cell monolayers for virus plaque counts. *J. Bacteriol.*, **78**, 596–597.

IBRAHIM, A. L. and P. C. LOH (1972): Comparison of the immunofluorescent-cell counting and plaque methods for the assay of vaccinia virus. *Appl. Microbiol.*, **23**, 214–217.

KELLY, R. K., B. W. SOUTER, and H. R. MILLER (1978): Fish cell lines: Comparison of CHSE-214, FHM, and RTG-2 in assaying IHN and IPN viruses. *J. Fish. Res. Board Can.*, **35**, 1009–1011.

KIMURA, T., M. YOSHIMIZU, M. TANAKA, and H. SANNOHE (1981a): Studies on a new virus (OMV) from *Oncorhynchus masou*—I. Characteristics and pathogenicity. *Fish Pathology*, **15**, 143–147.

KIMURA, T., M. YOSHIMIZU, and M. TANAKA (1981b): Studies on a new virus (OMV) from *Oncorhynchus masou*—II. Oncogenic nature. *Fish Pathology*, **15**, 149–153.

KLEEMAN, K. T., J. L. FRYER, and K. S. PILCHE (1970): Observed differences in CO_2 requiremenst between mammalian and salmonid fish cell lines. *J. Cell Biol.*, **47**, 796–798.

LEONG, J. C., J. L. FENDRICK, S. YOUNGMAN, and A. LEE (1981): Effect of polybrene on the infectivity of infectious haematopoietic necrosis virus in tissue culture. *J. Fish Diseases*, **4**, 335–344.

MACDONALD, R. D. (1978): Ringed plaque formation in infectious pancreatic necrosis virus correlates with defective interfering particle production. *J. Gen. Virol.*, **41**, 623–628.

MIRCHAMSY, H. and F. RAPP (1968): A new overlay for plaquing animal viruses. *Proc. Soc. Exp. Biol. Med.*, **129**, 13–17.

MORENS, D. M., S. B. HALSTEAD, and L. K. LARSENG (1985): Comparison of denge virus plaque reduction neutralization by macro and 'semi-micro' methods in LLC-MK2 cells. *Microbiol. Immunol.*, **29**, 1197–1205.

MOSS, L. H. and M. GRAVELL (1969): Ultrastructure and sequential development of infectious pancreatic necrosis virus. *J. Virol.*, **3**, 52–58.

NICHOLSON, B. L., G. W. THORNE, C. JANICKI, and A. HANSON (1979): Studies on a host range variant from different isolates of infectious pancreatic necrosis virus (IPNV). *J. Fish Diseases*, **2**, 367–379.

ROBIN, J., C. LARIVIERE-DURAND, and L. BERTHIAUME (1982): Plaque assay of bluegill virus using a methylcellulose overlay. *J. Virol. Methods*, **5**, 351–354.

ROIZMAN, B., C. E. CARMICHAEL, F. DEINHARDT, G. DE-THE, A. J. NAHMIAS, W. PLOWRIGHT, F. RAPP, P. SHELDRICK, M. TAKAHASHI, and K. WOLF (1981): Herpesviridae, definition, provisional nomenclature and taxonomy. *Intervirology*, **16**, 201–217.

ROSSI, C. R. and A. M. WATRACH (1970): Studies of laryngotracheitis virus in avian tissue cultures III. Enhancement of infectivity by diethylaminoethyl-dextran. *Appl. Microbiol.*, **19**, 932–936.

SCHERRER, R. and J. COHEN (1975): Studies on infectious pancreatic necrosis virus interactions with RTG-2 and FHM cells: Selection of a variant virus-type in FHM cells. *J. Gen. Virol.*, **28**, 9–20.

TOMASEC, J. and N. FIJAN (1971): Virusne bolesti riba (viral diseases of fish). Final Report on Research under a Part of Project 6n/1966. Zagreb, 29 pp.

WOLF, K. and M. C. QUIMBY (1962): Established eurythermic line of fish cells *in vitro*. *Science*, **135**, 1065–1066.

WOLF, K. and M. C. QUIMBY (1966): Lymphocystis virus: Isolation and propagation in centrarchid fish cell lines. *Science*, **151**, 1004–1005.

WOLF, K. and M. C. QUIMBY (1969): Fish cell and tissue culture. *In*: Fish Physiology (Hoar, W. S. and Randall, D. J., eds.) pp. 253–305. Academic Press, New York.

WOLF, K. and M. C. QUIMBY (1973): Fish viruses: Buffers and methods for plaquing eight agents under normal atmosphere. *Appl. Microbiol.*, **25**, 659–664.

YOSHIMIZU, M., M. TANAKA, and T. KIMURA (1987): *Oncorhynchus masou* virus (OMV): Incidence of tumor development among experimentally infected representative salmonid species. *Fish Pathology*, **22**, 7–10.