Characteristics of A New Reo-like Virus Isolated from Landlocked Salmon (*Oncorhynchus masou Brevoort*)

Ya-Li HSU, Bor-Shiun CHEN, and Jen-Leih WU

*Institute of Zoology, Academia Sinica, Nankaang, Taipei, Taiwan,
11529, R.O.C.*

(Received November 14, 1988)

A new reo-like virus was isolated from landlocked salmon around the Wu-Lin farm in the upper basin of Ta-Chia river, and designated as LSV (landlocked salmon virus). It has shown typical plaque-like syncytial CPE in tissue culture, and replicated very well in AS, BF-2, BB, CCO and CHSE-214 cell lines. But RTG-2 and TO-2 cells produced little virus. The optimal growth temperature of LSV in CHSE-214 is 20°C. The typical double-layered capsid of icosahedral virion with a 78 nm diameter was shown in electron micrography. The buoyant density of LSV in CsCl is 1.365 g/ml. Those are typical characteristics of Reoviridae. However, LSV has 11 segmented ds RNA genomes and 5 major virion proteins, has aquatic animal hosts which are characteristics of aquareovirus, but is different from viruses of the defined six genera of Reoviridae. Compared to the electrophoretotype of RNA genomes and virion proteins of LSV, CSV, CRV, GSV, and 13P2, the RNA pattern of LSV is closer to that of CSV, but the virion protein pattern is closer to that of 13P2. However, each aquareovirus has its own unique RNA and virion protein pattern. These results indicate that LSV is a newly discovered virus and the first aquareovirus isolated in Taiwan.

The landlocked salmon (*Oncorhynchus masou* BREVOORT) was found near the Wu-Lin farm in the upper basin of Ta-Chia river of Taiwan. It was first described by a technician in Taiwan in 1917 (LIN, 1986). This species is now near extinction. In 1984, our government initiated a culture center and a program to protect the landlocked salmon. The artificial hatchery was started and supported by the Lu-Kung Fisheries branch in 1985 (Hsu *et al.*, 1987). The mortality rate of these fries was then about 90%. Most died in fry or egg stages. Our laboratory began to investigate the disease problem of the landlocked salmon in order to understand the reasons for the high mortality. Since regulations prohibit killing live fish of this species, we can only study the disease problem by using these moribund eggs and fish (Hsu *et al.*, 1987). The overgrowth of intestinal bacteria and cytotoxicity of the dead bodies from moribund fish covered the pathogens, making study difficult. However, from 7 adult fish, 357 eggs and 193 fries, one reo-like virus was isolated from a 14.2 cm length fish. This isolate was designated landlocked salmon virus (LSV).

Nine reo-like viruses isolated from fish and shellfish have been reported; eight from the fish hosts, golden shiner (*Notemigonus crysoleucas*, PLUMB *et al.*, 1979), chum salmon (*Oncorhynchus keta*, WINTON, 1981), channel catfish (*Ictalurus punctatus*, HEDRICK *et al.*, 1984), grass carp reovirus (*Ctenopharyngodon idellus*, CHEN & JIANG, 1984a, b), tench (*Tinca tinca*, AHNE and KOLBL, 1987), chub (*Leuciscus cephalus*, AHNE and KOLBL, 1987), fency carp (*Cyprinus carpio*, SANO and FUKUDA, 1987), Japanese eel (*Anguilla japonica*, SANO and FUKUDA, 1987) and one from the American oyster (*Crassostrea virginica*, MEYERS, 1979). These viruses were compared (WINTON *et al.*, 1987). In this paper are described the characteristics of LSV as compared with the above mentioned 4 reolike viruses (CSV, GSV, CRV and 13P2) by their morphology, and an analysis of SDS-PAGE of the major virion protein and 11 segmented RNA genome patterns. The pathogenicity of LSV was also discussed.

Materials and Methods

Viruses

Golden shiner virus (GSV), channel catfish reovirus (CRV), and American oyster virus (13p2) were kindly given by Dr. R. P. HEDRICK, University of California, Davis, U.S.A. and chum salmon virus (CSV) was given by Dr. J. R. Winton, National Fisheries Research Center, Seattle, WA.. Landlocked salmon virus (LSV) isolated from diseased fish was plaque-purified three times prior to use in our morphological and biochemical studies. Human reovirus type 3 (Dearing strain) was provided by Dr. C. S. Lai, National Taiwan University, Taipei, Taiwan, R.O.C.

Cell lines

CHSE-214 cells (NIMS *et al.*, 1970), BF-2 (WOLF and QUIMBY, 1966), BB (WOLF and QUIMBY, 1969), CCO (BOWSER and PLUMB, 1980), AS (NICHOLSON and BYRNE, 1973), EPC (TOMASEC and FIJAN, 1971), FHM (GRAVELL and MALSBERGER, 1965), GK (NICHOLSON *et al.*, 1987), MHR, PH, PL (NICHOLSON *et al.*, 1987), RTG-2 (WOLF and QUIMBY, 1962) and TO-2 (CHEN *et al.*, 1983) were used to test the replication of LSV. CHSE-214 cells were used to propagate and assay the CSV, GSV, 13P2 and LSV isolates. The channel catfish ovary (CCO) cells were used for the multiplication of CRV.

All cell lines were grown as monolayer cultures using minimum essential medium (MEM) with Earle's salts (Gibco) and 10% (MEM-10) fetal calf serum (Flow lab., U.S.A.), 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml gentamycin (Gibco), 2.5 µg/ml fungizone (Squibb and Sons Inc., USA) and 5 ml of 7.5% NaHCO₃. CHSE-214 and AS were incubated at 20°C, the other cell lines at 25°C. Only TO-2 cells were cultured in L-15 medium.

Virus Isolation

The tissues of liver, kidney, spleen, pancreas, heart, and gill were homogenized in a polytron (Kine matic, Switzerland) with 10 vol (V/W) of MEM containing double strength antibiotics (WU *et al.*, 1987). The homogenate was centrifuged and the supernatant was filtered and then inoculated onto the monolayers of BF-2, CCO, CHSE-214 and AS cell lines. The inoculated

culture plates were examined daily. When extensive cytopathic effect was observed, the medium was filtered and infected again with a fresh culture.

Virus assay

The virus titers were determined by TCID₅₀ in 96 well microplates (Greiner, W. Germany) and the endpoints were calculated according to REED and MUENCH (1938).

Virus purification

The virus purification procedure was adapted from a modified method described by WINTON *et al.* (1987). The harvested virus suspension was centrifuged at 100,000×g for 1 hr. The virus pellet was resuspended in SSC (0.15 M-sodium chloride, 0.015 M-sodium citrate, pH 7.4) and extracted with Freon 113 (1, 1, 2-trichloro-1, 2, 2-trifluoroethane). The aqueous phase was placed onto a continuous sucrose gradient (15 to 50% sucrose in SSC) and centrifuged at 100,000×g for 2 hrs. The virus band was collected and loaded onto a three step CsCl gradient (2.0 ml of 40% CsCl, 1.5 ml of 30% CsCl, and 1.0 ml of 20% CsCl in SSC), and centrifuged at 115,000×g for 16 hrs. The virus band was withdrawn and resuspended in SSC and pelleted by centrifugation at 115,000×g for 1 hr.

Electron microscopy

A drop of purified virus in distilled water was placed onto a Formvar coated grid. The unbuffered 2% phosphotungstic acid (adjusted with NaOH to pH 7.0) was added and stained for 1 min. The excess solution was removed with a filter paper wedge and the virions were examined with a Hitach electron microscope.

Density of virions

Purified virus was placed onto the top of a three step CsCl gradient and centrifuged for 16 hrs at 115,000×g at 4°C. Fractions were collected, and the density of each fraction was determined using an Abbe's refractometer (Bausch and Lomb).

Viral RNA gel electrophoresis

When extensive CPE was observed in the infected CHSE-214 cells of 25 cm² flask (CCO for

FIG. 1. The cytopathic effect of LSV on the CHSE-214 cells. A. Normal CHSE-214 cells. B. The typical plaque-like syncytial CPE in CHSE-214 cells. C & D. Extensive CPE of LSV in CHSE-214 cells.

CRV), the virus suspension was mixed with sarkosyl to a final concentration of 0.1% and centrifuged at $130,000 \times g$ for 1 hr at 4 C. The pellet was incubated with 0.5% SDS and 200 $\mu\text{g}/\text{ml}$ proteinase K in a TNE buffer (0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.3) at 37 C overnight. Then an equal volume of 2 $\times$ SDS sample buffer was added and boiled for 2 min (Hsu *et al.*, 1988; Hedrick *et al.*, 1983). For the RNA pattern, 10% polyacrylamide gel electrophoresis was used, and 5 mA/gel was conducted for 40 hrs. The gel was stained with silver nitrate.

Virion protein gel electrophoresis

Purified virus was mixed with protein sample buffer (Hsu, *et al.*, 1985) and boiled for 3 min, and applied onto the 10% SDS-polyacrylamide gel (Laemmli, 1970). After electrophoresis for 4 hrs at 20 mA, the gel was fixed and stained with silver nitrate (Hsu, *et al.*, 1986).

Silver stain

The silver stain procedure for nucleic acid was modified from Goldman's and Guillemette's (Goldman *et al.*, 1980; Guillemette and Lewis, 1983) methods. After electrophoresis for RNA, the gel was fixed in 50% methanol and 12% acetic acid for 3 min. The SDS in the gel was removed

Table 1. The multiplication of landlocked salmon virus (LSV) in selected fish cell lines at 18°C

Cell line	Abbreviation	TCID ₅₀ /ml
Atlantic salmon	AS	2.2×10^5
Bluegill fry	BF-2	7.0×10^5
Brown bullhead	BB	5.0×10^7
Channel catfish ovary	CCO	1.0×10^8
Chinook salmon embryo	CHSE-214	1.0×10^7
Epithelioma papillosum cyprini	EPC	3.2×10^4
Fathhead minnow	FHM	5.0×10^3
Grouper kidney	GK	1.6×10^4
Milkfish heart	MHR	6.8×10^5
Perch heart	PH	6.8×10^4
Perch liver	PL	4.7×10^6
Rainbow trout gonad	RTG-2	6.8×10^3
Tilapia ovary	TO-2	3.2×10^2

Table 2. Effect of temperature on landlocked salmon virus (LSV) multiplication in BF-2 cells

Temperature	10°C	15°C	13°C	20°C	22°C	25°C	30°C
TCID ₅₀ /ml	4.3×10^4	7.9×10^6	7.9×10^6	2.0×10^7	1.3×10^7	1.4×10^6	4.0×10^4

by thrice washing with 10% (v/v) ethanol and 5% (v/v) acetic acid, each time for 10 min. Then the gel was soaked in 0.1% CTAB (cetyltrimethylammonium bromide) for 30 min, and washed with distilled water for 30 min. The gel was changed into 0.3% ammonia and shaken for 15 min. Then the ammoniacal silver solution (1.6% silver nitrate, 0.12% ammonia, 4 mM NaOH) was replaced and shaken for 15 min. The gel was rinsed with distilled water within 15 sec. The developed solution (2% sodium carbonate, 0.02% formaldehyde) was added quickly and shaken until the RNA band appeared. The development was stopped by adding 0.25% nitric acid for 15 min, and then the gel was rinsed with water and dried for storage.

Results

Virus isolation

Only one virus was isolated from the liver and gills of one diseased fish. This fish showed necrosis of the pelvic fin and internal organs (Hsu, *et al.*, 1978). After inoculation of tissue homogenate on different monolayers, such as AS, BF-2, CCO, and CHSE-214, these cells were incubated at 20°C or 25°C. After 14 days postinoculation, the typical cytopathic effect of these cells showed

the plaque-like syncytia. The fusion and lysis of these cells became more serious at longer period of incubation (Fig. 1). This reo-like virus isolate was designated landlocked salmon virus (LSV).

Host range

After three consecutive plaque assays, the LSV virus was cultured, and the host range of LSV was determined by 13 fish cell lines. The results are shown in Table 1. LSV replicated very well in AS, BF-2, BB, CCO, and CHSE-214 cell lines. Limited replication occurred in EPC, FHM, GK, MHR, PH and PL cells, while RTG-2 and TO-2 were refractory to produce CPE and little virus. LSV cannot replicate in BHK-21 mammalian cells and human reovirus (type 3) can not replicate in CHSE-214 fish cells.

Optimal growth temperature

Virus fluid (0.5 ml) with 10 TCID₅₀ was separately placed onto a monolayer of BF-2 cells in 25 cm² flasks. Two flask were incubated at each temperature; at 10, 15, 18, 20, 22, 25 and 30°C. When extensive CPE was observed, the virus suspension was centrifuged and the supernatant was titrated by TCID₅₀ method (REED and MUENCH, 1938). The results are shown in Table 2 and Fig. 2. The optimal temperature for LSV multiplication was

Fig. 2. Effect of temperature on the multiplication of LSV in BF-2 cells.

Fig. 3. Electron micrograph of negatively stained LSV

20°C LSV can replicate very well in 15 to 25°C, but not at 10 or 30°C

Virion density and morphology

After centrifugation in CsCl gradient, the virus band was collected, and the buoyant density of the complete particles was measured in CsCl, and found to be 1.365 g/ml. The purified virus was negatively stained, and under EM observation, the virions revealed particles with icosahedral symmetry and a double capsid shell of about 78 nm mean diameter (Fig 3). Twenty peripheral capsomeres were present around the periphery of the outer capsid shell. These morphological features are characteristics of members of the Reoviridae.

Fig. 4. Electrophoretotypes of RNA genomes of LSV, CSV, Reo-3, GSV and CRV

SDS-PAGE analysis of RNA genome

Electrophoresis of RNA genome by using 10% SDS-PAGE, this silver-stained gel revealed that each of the aquareoviruses, LSV, CSV, GSV and CRV, had a genome composed of 11- segmented ds RNA (Fig 4). The molecular weight of all these RNA segments fell into three size classes that ranged from 2.5×10^6 to 0.31×10^6 daltons (Table 3). There were three large (L1, L2, and L3), three medium (M1, M2, and M3), and five small segments (S1, S2, S3, S4, and S5) in each genome (Fig 4) (WINTON *et al.*, 1983). However, reovirus type 3 (Reo-3 in Fig. 4) was quite different; it had four instead of five small RNA segments. Each aquareovirus had its own unique RNA electrophoretotype (Fig 4), but the total genome of each was approximately 15×10^6 daltons, which was consistent with the values of other reoviruses. However, the RNA patterns of these aquareoviruses were unlike those reported for members of the six genera currently defined with the Reoviridae (JOKLIK, 1981, 1983a; 1983b; WINTON, *et al.*, 1987).

Table 3. Estimates of molecular weight of RNA segments of five reoviruses from aquatic animals compared to reovirus type 3 ($\text{MW} \times 10^{-6}$)

Segment	RNA molecular weight ($\times 10^{-6}$)					
	13P2*	CSV	LSV	GSV	CRV	Reo-3
L1	2.40	2.40	2.50	2.50	2.40	2.50
L2	2.30	2.40	2.40	2.40	2.40	2.40
L3	2.30	2.30	2.35	2.20	2.30	2.30
M1	1.90	1.80	1.75	1.60	1.60	1.60
M2	1.70	1.70	1.70	1.50	1.50	1.60
M3	1.40	1.50	1.50	1.40	1.30	1.40
S1	0.85	0.88	0.90	0.90	0.90	0.92
S2	0.80	0.85	0.89	0.80	0.88	0.76
S2	0.57	0.61	0.60	0.66	0.61	0.64
S4	0.47	0.47	0.50	0.40	0.41	0.61
S5	0.35	0.31	0.31	0.35	0.40	none
Total	15.04	15.22	15.71	14.71	14.70	14.93

* From WINTON *et al.* (1987).**Table 4.** Estimates of molecular weight of the major structural virion proteins of five reoviruses from aquatic animals

Polypeptide	(M.W. $\times 10^{-3}$)				
	LSV	13P2	CSV	GSV	CRV
λ_1	139	135	137	137	132
λ_2	130	128	126	130	130
μ_1	69	70	68	63	70
σ_1	44	45	45	43	43
σ_2	34	33	33	36	32

The RNA pattern of LSV was closer to that of CSV, but quite different. The major difference between RNA segments of LSV and CSV was in S1, S2, and S3 (Fig. 4). Compared with the estimated molecular weight of each RNA of aquareovirus, including 13P2 (WINTON *et al.*, 1987) (Table 3), we found that each virus had a unique pattern, which indicated that LSV was a new aquareovirus.

SDS-PAGE analysis of virion polypeptides

A comparison of virion proteins of LSV, CSV, CRV, GSV and 13P2 was made by silver-stained 10% SDS-PAGE as shown in Fig. 5. The estimated molecular weight of each polypeptide is listed in Table 4. These aquareoviruses had two large (λ_1 and λ_2), one medium (μ_1) and two small virion proteins (σ_1 and σ_2) ranging from 139,000 to 32,000. According to Fig. 5, the peptide pattern of LSV was closer to that of

Fig. 5. Electrophoretotypes of major virion proteins of CSV, CRV, GSV, 13P2, and LSV.

13P2, but their μ_1 , λ_1 and λ_2 were lightly different (Table 4). The virion protein pattern of LSV was unique and different from that of CSV, GSV, CRV and 13P2. This was another index that LSV was a new aquareovirus isolate.

Discussion

The first reo-like virus isolated from golden shiner (GSV) was reported by PLUMB *et al.* (1979). Schwedler and Plumb (1980) indicated that this GSV was clearly different from the birnaviruses both in particle morphology and serological characteristics. In 1981, WINTON *et al.* also described a chum salmon virus (CSV) that shared physical properties with GSV, suggesting the two viruses were related, and also revealed that CSV contained 11 segments of ds RNA (WINTON *et al.*, 1983). AMEND *et al.* in 1984 reported that a channel catfish virus (CRV) shares physical and morphological properties with both GSV and CSV and that the antisera of CRV also neutralizes CSV and GSV. In 1987, WINTON *et al.* compared the morphological and biochemical properties of these four reo-like viruses (CSV, CRV, GSV and 13P2) isolated from aquatic animals, and indicated that these viruses with common characters should be categorized to as a common group. CHEN and JIANG in 1984 (a and b) described and characterized a virus isolated from the hemorrhagic grass carp and identified this virus as a reovirus which was tentatively designated as herpesvirus of grass carp in 1980. SANO and FUKUDA indicated reo-like viruses have been isolated from fancy carp and Japanese eel from Tokyo university of Fisheries in 1987. At the same year, Ahne and Kolbl reported that two viruses have been isolated from tench (*Tinca tinca*) and chub (*Leuciscus cephalus*). They have a double capsid and a size of 70–75 nm with a 11 segments of ds RNA genome.

In our laboratory, a routine rapid RNA gel electrophoresis was analyzed for confirmation and epizootiological studies of IPNV isolates. When a typical plaque-like syncytial CPE of a isolate appeared and its RNA patterns showed a reo-like virus, we started to culture this LSV in Baby hamster kidney (BHK-21) mammalian cells, and none of them grew. At the same time, we cultured human reovirus type 3 in CHSE-214 fish

cell line. It also failed. From these results, we know that LSV and Reo-3 have quite different host and multiplication temperatures. However, LSV have 11 segments of ds RNA and 78 nm diameter icosahedron with double-layered capsid which expresses a typical reo-like virus. Compared to the electrophoretotype of RNA and virion protein patterns of LSV with those of CSV, CRV, GSV and 13P2 (Figs. 4, 5, and Tables 3 and 4), it is obvious that each aquareovirus had a unique RNA and protein pattern. The RNA pattern of LSV is closer to that of CSV than of 13P2, and the virion protein pattern is closer to that of 13P2. These results indicated that LSV is a new isolate of aquareovirus. However, given such a low frequency of viral isolation and slow cytopathic effect in tissue culture, it seems reasonable to conclude that LSV is not a contagious and virulent pathogen for landlocked salmon. But it must widespread enough in aquatic animals that we should screen this aquareovirus carefully. Recently, our laboratory also obtained one isolate from rainbow trout and one from hard clam, only the culture pond of hard clam had mortality (data not shown). This is correlated with the reports that the pathogenicity of these aquareoviruses except for the 13P2 virus (MEYERS, 1980) in fish is relatively low.

MATTHEWS (1982) described that the Reoviridae family is composed of viruses with 10 to 12 segments of ds RNA and double-layered icosahedral virion. JOKLIK (1983a) also used genome numbers, particle structure, host range, and sensitivity to physical and chemical treatments to categorize the Reoviridae into six genera. Genus Orthoreovirus, Fijivirus, Orbivirus and Cypovirus have 10 segments of ds RNA; Phytoreovirus have 12 segments of ds RNA. Only Rotavirus has 11 segments of ds RNA. However, the viruses compared in this study showed electrophoretotypes of RNA different from that of Rotavirus, and they have different aquatic animal hosts. In addition, Rotavirus RNA revealed no detectable homology with P-labelled CSV probe at 50% stringency (WINTON *et al.*, 1987). Serological test revealed that the infectivity of CSV could not be neutralized by antisera against the three mammalian reovirus serotypes (WINTON, 1981) and that 13P2 could not be neutralized by antisera against Feline reovirus

type 3, canine reovirus type 1 or chicken FDO reovirus (MEYERS, 1979). Based on the host range, optimal growth temperature, RNA segment patterns, and serological tests, the viruses in this study should be categorized as Reoviridae but probably in different genera aquareovirus.

Acknowledgements

We wish to thank Dr. R. P. HEDRICK and Dr. J. R. WINTON for sending isolates of aquareoviruses. This work was supported by a grant from the National Science Council (NSC-77-0211-B001-39), and the Council of Agriculture (77-11.2-Forestry-22(4)).

References

- AHNE, W. and O. KOLBL (1987): Occurrence of reoviruses in European cyprinid fishes (*Tinca tinca* Lin.; *Leuciscus cephalus* Lin.) *J. Appl. Ichthyol.*, **3**, 139-141.
- AMEND, D. F., T. McDOWELL and R. P. HEDRICK (1984): Characteristic of a previously unidentified virus from channel catfish (*Ictalurus punctatus*). *Can. J. Fish. & Aqua. Sci.*, **41**, 807-811.
- BOWSER, P. R. and J. A. PLUMB (1980): Growth rates of a new cell line from channel catfish ovary and channel catfish virus replication at different temperatures. *Can. J. Fish. Aqua. Sci.*, **37**, 871-873.
- CHEN, S. N., S. C. CHI, Y. UENO and G. H. KOU. (1983): A cell line derived from tilapia ovary. *Fish Path.*, **18**, 13-18.
- CHEN, Y. and JIANG (1984a): Morphological and Physico-chemical characterization of the hemorrhagic virus of grass carp. *Kexue Tongbao*, **29**, 832-835.
- CHEN, Y. and JIANG (1984b). Morphological physico-chemical characterization of the hemorrhagic virus of grass carp. Sixth International Congress of Virology. Abstracts W37-1, pp. 373.
- GOLDMAN, D., S. A. SEDMAN and M. H. EBBERT (1980): Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science*, **211**, 1437-1438.
- GRAVELL, M., and R. G. MALSBERGER (1965): A permanent cell line from the fathead minnow (*Pimephales promelas*). *Ann. N. Y. Acad. Sci.*, **126**, 555-565.
- GUILLEMETTE, J. G. and P. N. LEWIS (1983): Detection of subnanogram quantities of DNA and RNA on native and denaturing polyacrylamide and agarose gels by silver staining. *Electrophoresis*, **4**, 92-94.
- HEDRICK, R. P., J. L. FRYER, S. N. CHEN and G. H. KOU (1983): Characteristics of four Birnaviruses isolated from fish in Taiwan. *Fish Pathol.*, **18**(2), 91-97.
- HEDRICK, R. P., R. ROSEMARK, D. AROUSTEIN, J. R. WINTON, T. MCCDOWELL and D. F. AMEND (1984): Characteristics of a new reovirus from channel catfish (*Ictalurus punctatus*). *J. Gen. Virol.*, **65**, 1527-1534.
- HSU, Y. L., B. S. CHEN and J. L. WU (1988). The comparison of RNA and protein gel patterns of Infectious pancreatic necrosis virus from eel and rainbow trout. *J. Gen. Virol.* (submitted).
- HSU, Y. L., B. S. CHEN, C. Y. CHIEN, J. L. WU, Y. S. CHOW, and K. S. CHANG. (1987): The study of fungi, bacteria and viruses of *Oncorhynchus masou* (Brevoort) I. Annual report of Ecology. no. 009, 18 pp.
- HSU, Y. L., H. M. ENGELKING, and J. C. LEONG. (1986): Occurrence of different types of infectious hematopoietic necrosis virus in Fish. *Appl. Environ. Microbiol.*, **52**, 1353-1361.
- HSU, Y. L., H. M. ENGELKING, and J. C. LEONG. (1985): Analysis of the quantity and synthesis of the virion proteins of infectious hematopoietic necrosis virus. *Fish Path.*, **20**, 331-338.
- JOKLIK, W. K. (1981): Structure and function of the reovirus genome. *Microbiol. Reviews*, **45**, 483-501.
- JOKLIK, W. K. (1983a): The members of the family Reoviridae. In "The Reoviridae", ed. W. K. Joklik, N. Y. Plenum Press, pp. 1-7.
- JOKLIK, W. K. (1983b): The reovirus particle. In "The Reoviridae", ed. W. K. Joklik, N. Y. Plenum Press, pp. 9-78.
- LAEMMLI, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature*, **227**, 680-685.
- LIN, Yao-Sung (1986): Landlocked salmon in Taiwan highland. In: Memoir of Nature, Endangered and Rare plant/Animal species and landscape conservation (II). COA Forestry Series No. 9, p. 1-13.
- MATTHEWS, R. E. F. (1982): Classification and nomenclature of viruses. *Intervirology*, **17**, 1-199.
- MEYERS, T. R. (1979): A reo-like virus isolated from juvenile American oysters (*Crassostrea virginica*). *J. Gen. Virol.*, **43**, 203-212.
- MEYERS, T. R. (1980): Experimental pathogenicity of reovirus 13P2 in juvenile American oysters (*Crassostrea virginica*) (Gmelin) and in bluegill fingerlings (*Lepomis macrochirus*) (Rafinesque). *J. Fish Dis.*, **3**, 187-201.
- NICHOLSON, B. L. and C. BYRNE (1973): An established cell line from the Atlantic salmon (*Salmo salar*). *J. Fish. Res. Board Can.*, **30**, 913-916.
- NICHOLSON, B. L., D. J. DANNER and J. L. WU. (1987):

- Three new continuous cell lines from marine fishes of Asia. *In Vitro*, **23**, 199–204.
- NIMS, L., J. L. FRYER and K. S. PILCHER (1970): Studies of replication of four selected viruses in two cell lines derived from salmonid fish. *Proc. Soc. Exp. Biol. Med.*, **135**, 6–12.
- PLUMB, J. A., P. R. BOWSER, J. M. GRIZZLE and A. J. MITCHELL. (1979): Fish viruses: A double-stranded RNA icosahedral virus from a North American cyprinid. *J. Fish. Res. Board Can.* **36**, 1390–1394.
- REED, L. J. and H. MUENCH (1938): A simple method of estimating fifty percent endpoints. *Amer. J. Hyg.*, **27**, 493–497.
- SANO, T. and H. FUKUDA (1987): Principal Microbial diseases of mariculture in Japan. *Aquaculture*, **67**, 59–69.
- SCHWEDLER, T. E., and J. A. PLUMB (1980): Fish viruses: Serological comparison of golden shiner and infectious pancreatic necrosis viruses. *J. Wildlife Dis.*, **16**, 597–599.
- TOMASEC, J. and N. FIJAN (1971): Virusne balestiriba (viral diseases of fish). Final report on research under a part of project, 6n/1966, Zagreb.
- WINTON, J. R. (1981): Isolation and characterization of a new reovirus from chum salmon (*Oncorhynchus keta*). Ph. D. Thesis, Oregon State University.
- WINTON, J. R., C. N. LANNAN and J. L. FRYER (1983): Further characterization of a new reovirus of poikilothermic vertebrates. In “Double-stranded RNA viruses”, pp. 231–236, ed. R. W. Compans and D. H. L. Bishop, N. Y. Elsevier.
- WINTON, J. R., C. N. LANNAN, J. L. FRYER, R. P. HEDRICK, T. R. MEYERS, J. A. PLUMB, and T. YAMAMOTO (1987): Morphological and Biochemical properties of four members of a novel group of reoviruses isolated from aquatic animals. *J. Gen. Virol.*, **68**, 353–364.
- WOLF, K. and M. C. QUIMBY (1962): Established eurythermic line of fish cells *in vitro*. *Science*, **135**, 1065–1066.
- WOLF, K., 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. pp. 253–305, In “Fish Physiology” ed. W. S. Hoar & D. J. Randall, Academic Press.
- WU, J. L., C. Y. CHANG and Y. L. HSU (1987): Characteristics of infectious pancreatic necrosis like virus isolated from Japanese eel (*Anguilla japonica*). *Bull. Inst. Zool. Academia Sinica*, **26**, 201–214.