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Author(s)	Nishizawa, T.; Yoshimizu, M.; Winton, J.; Kimura, T.
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## COMPARISON OF STRUCTURAL PROTEIN ON FISH RHABDOVIRUSES

T.NISHIZAWA\*1, M.YOSHIMIZU, J.WINTON\*2, and T.KIMURA

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

### INTRODUCTION

Two major genera of the family Rhabdoviridae, *Vesiculovirus* and *Lyssavirus*, have been described (Matthews 1982, Wagner 1987). Vesicular stomatitis virus (VSV), the prototype *Vesiculovirus*, has a virion composed of 5 structural proteins; polymerase (L), glycoprotein (G), nucleocapsid protein (N), a phosphoprotein (P, formerly N<sub>s</sub>), and matrix protein (M)(Kang & Prevec 1971, Knipe et al. 1975, Wagner 1987). The virion of rabies virus, the prototype *Lyssavirus*, is composed of L, G, N, and two matrix proteins (M1 and M2) (Madore & England 1977, Wagner 1987). Among the rhabdoviruses of fish, pike fry rhabdovirus (PFR), spring viremia of carp virus (SVCV), eel virus European X (EVEX), and eel virus of America (EVA) have been shown to be vesiculoviruses while infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and hirame rhabdovirus (HRV) are lyssaviruses (Hill et al. 1975, Lenoir & de Kinkelin 1975, de Kinkelin et al. 1980, Hill et al. 1980, Leong et al. 1981, Hsu et al. 1985, Lorenzen et al.1988, Kimura et al. 1986, Kimura et al.1989, Nishizawa et al. 1991a).

We used SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) to analyze and compare the five structural proteins of seven fish rhabdoviruses and compare their relative mobilities. Because IHNV, VHSV, and HRV were similar to each other by SDS-PAGE, we used 2-dimensional electrophoresis (O'Farrell 1975) to determine the isoelectric points of the structural proteins of these three viruses. A western blot analysis (Towbin et al.1979) was used to examine the antigenic relatedness of these structural proteins. We tested the stability of IHNV and HRV in the presence of Triton X-100 which has been shown to selectively remove structural proteins from the IHNV virion (McAllister & Wagner 1975). The results of our study indicate that HRV, IHNV, and VHSV belong to the same genus of *Lyssavirus*, however, they can be clearly distinguished from each other by the methods employed in this study.

### MATERIALS AND METHODS

#### Cell lines and viruses

Isolates of HRV (strain 8401H), IHNV (isolated from rainbow trout, Cultus Lake, Canada), VHSV (Strain F1), PFR, SVCV, EVEX, and EVA were used in this study. These were kindly provided by Dr.P.V.Jorgensen, State Serum Laboratory, Arhus, Denmark (VHSV), Dr. B.J. Hill, Fish Disease Laboratory,

\*1: Laboratory of Fish Pathology, Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima, 724 Japan,

\*2: National Fisheries Research Center, Building 204, Naval Station, Seattle, Washington, USA.

Ministry of Agriculture, Fisheries and Food, UK (IHNv and SVCV), Dr. P.de Kinkelin, Institute National de la Recherche Agronomique, France (PFR), and Dr.T.Sano, Laboratory of Fish pathology, Tokyo University of Fisheries, Japan (EVEX and EVA). For the replication of these viruses, The epithelioma papulosum cyprini (EPC; Fijian et al. 1983) cell line was used.

#### Purification of virus

Viral purification using sucrose gradient centrifugation of culture fluid was performed as described by Nishizawa et al. (1991a), while viral purification using cesium chloride (CsCl) was performed as described by Nishizawa et al. (1991b).

#### Electrophoresis of protein

SDS-PAGE analysis of virion proteins was performed according to the method of Laemmli (1970), and two-dimensional gel electrophoresis (2DE) was performed using a modified procedure of a commercial protocol (Pharmacia)(Nishizawa et al.1991a).

#### Antisera to virion proteins

After virion proteins were separated by SDS-PAGE, they were extracted from the gel and used as antigens for the production of anti-viral protein sera in rabbit.

#### Western blot analysis

Western blot analysis of virion protein was performed using a modified method of Towbin et al.(1979) as already described by Nishizawa et al. (1991a).

#### Treatment of virions with Triton X-100

Purified virions from sucrose gradient were suspended in 10 mM Tris buffer (pH 7.4) with or without 1% Triton X-100 containing 0, 0.43, or 0.72M NaCl. After incubation at 25 C for 60 min, the treated virions were pelleted by centrifugation (150,000g,60min), then the viral pellet was resuspended in SDS-PAGE sample buffer.

Purified virions from CsCl gradient were suspended in STE buffer(20 mM Tris,pH 7.4,100 mM NaCl,1mM EDTA) with 1% Triton X-100. After incubation at 25 C for 0, 5, 10, 20, 40, or 60 min, the virion suspension was mixed with same volume of SDS-PAGE sample buffer.

Preparation of SDS-PAGE gels and electrophoresis condition were the same as described.

## RESULTS

#### Structural proteins of HRV

The results of SDS-PAGE analysis of the structural proteins of HRV, IHNv, VHSV, PFR, SVCV, EVEX, and EVA are shown in Fig.1. The patterns of HRV, IHNv, and VHSV were similar to each other, but differed from those of PFR, SVCV, EVEX, and EVA. Differences were observed among HRV, IHNv, and VHSV in the relative mobilities of G, N, M1, and M2 proteins. The M2 protein of HRV was smaller, while the N protein was larger, than the corresponding proteins of IHNv or VHSV. The HRV M1 protein was larger than that of VHSV but about the same as that of IHNv. These results showed that HRV, IHNv, and VHSV were typical members of the genus of *Lyssavirus*, but clearly differing from each other.

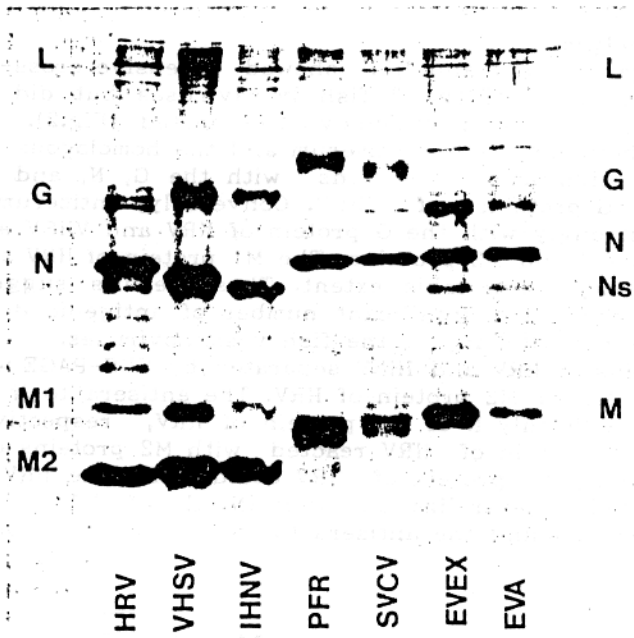


Fig.1. SDS-polyacrylamide gel electrophoresis patterns of the structural proteins of fish rhabdoviruses, HRV, VHSV, IHNV, PFR, SVCV, EVEX, and EVA.

#### Two-dimensional electrophoresis of HRV, IHNV, and VHSV

The results of the 2-dimensional gel electrophoresis of HRV, IHNV, and VHSV are shown in Table 2. Comparing HRV, IHNV, and VHSV, the pI values of the L, G, and N proteins were similar, but the pI values of the M1 and M2 proteins were quite different and could be used to distinguish HRV, IHNV, and VHSV from each other.

The 2DE gels revealed the M1 proteins of both HRV and IHNV migrated to form 2 separate spots which had the same molecular weight but different pIs (data not shown). The M1 protein of VHSV produced only one spot in the 2-dimensional electrophoresis gel.

Table 1. Iso electric point (pI) of the structural proteins of HRV, IHNV, and VHSV.

protein	HRV	IHNV	VHSV
large	6.2-6.7	6.0-7.0	6.0-6.8
glycoprotein	7.1-7.3	6.9-7.1	ND
nucleocapsid	5.9-6.2	5.9-6.2	5.9-6.2
matrix 1	7.3	7.8	8.5
	7.4	8.0	-
matrix 2	9.1-9.3	9.6-9.89	9.8

ND : not detected

### Western blot analysis

Antisera against HRV and IHNV showed extensive cross-reaction among the structural proteins of the 3 fish lyssaviruses but did not react with the proteins of the 4 fish vesiculoviruses tested (Fig.2). While staining was strongest between each antiserum and the homologous virus, anti-HRV serum showed significant reactions with the G, N, and M2 proteins of IHNV and the G proteins of VHSV. Conversely, antiserum against IHNV cross-reacted strongly with the G protein of HRV and VHSV and, to a lesser extent, with the N and M2 proteins. The M1 protein of HRV and IHNV did not cross-react to any detectable extent. These results suggest that HRV, IHNV, and VHSV share a significant number of antigenic determinants with each other, but not with any of the fish vesiculoviruses.

Virion proteins of HRV and IHNV separated by SDS-PAGE were stained with antiserum to N, M1, or M2 protein of HRV. The antisera to N and M1 protein of HRV reacted with only N or M1 protein of HRV, respectively, while the antiserum to M2 protein of HRV reacted with M2 proteins of both HRV and IHNV. Antiserum to M1 protein of HRV could neutralize HRV infectivity and had an estimated neutralization titer (ND<sub>50</sub>) of 1:15. No neutralization titer was detected using the antisera to N or M2 protein of HRV.

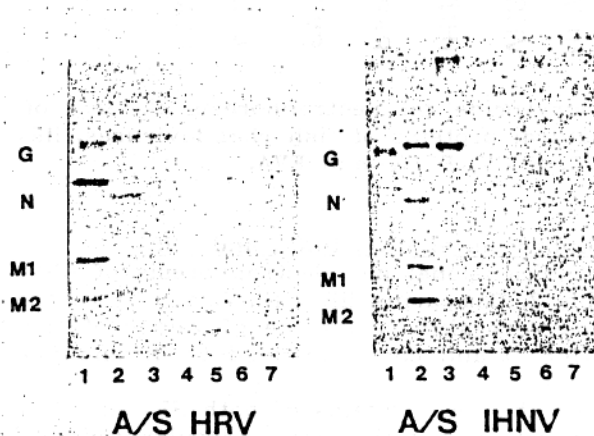


Fig.2. Western blot analysis of the structural proteins of fish rhabdoviruses stained with antiserum against HRV or IHNV. 1; HRV, 2; IHNV, 3; VHSV, 4; PFR, 5; SCVC, 6; EVEX, 7; EXA.

### Treatment of viruses with Triton X-100

We tested the effect of Triton X-100 on the stability of the HRV and IHNV virions (Fig. 3). The G protein of IHNV was readily removed by Triton X-100 and both the M1 and M2 proteins were removed by Triton X-100 containing 0.43 M or 0.72 M NaCl. However, the structural proteins of HRV were much more stable under the same conditions. The results of SDS-PAGE analysis of HRV virions purified using CsCl and treated with Triton X-100 are shown in Fig. 4. A gradual decrease in the amount of M1 protein was observed for HRV virions treated with Triton X-100. A new protein with estimated molecular weight of 10 KDa appeared and the amount of this protein increased over time. The same reaction was observed with M1 protein of IHNV, however, estimated molecular weight of the newly appearing protein was about 21 KDa (data not shown). These results suggest the newly appearing protein was produced by processing of M1 protein.

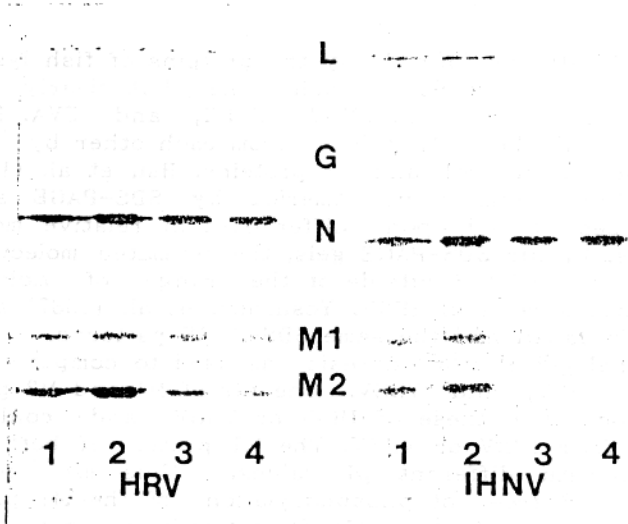


Fig.3. SDS-PAGE patterns of HRV and IHNV treated with Triton X-100 under the different concentration of NaCl. 1; non treatment, 2; Triton X-100 without NaCl, 3; Triton X-100 with 0.42M NaCl, 4; Triton X-100 with 0.72M NaCl

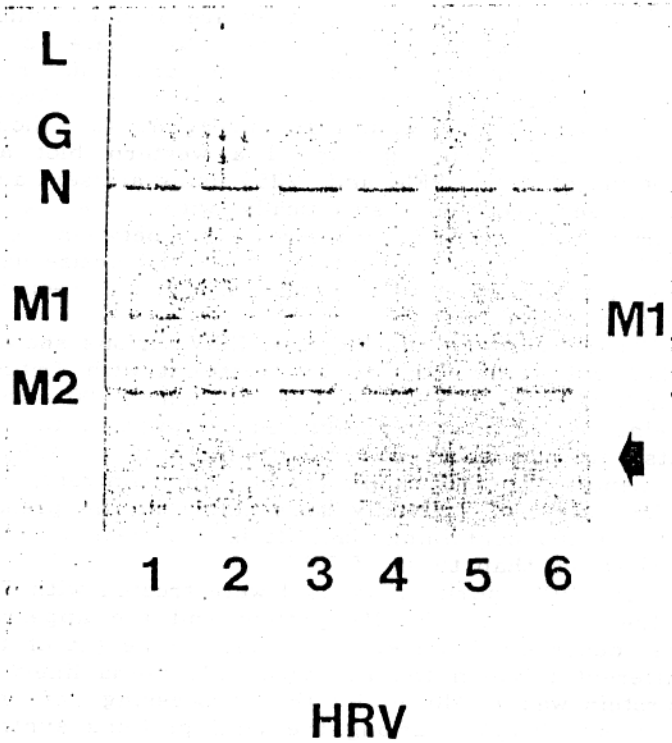


Fig.4. SDS-PAGE pattern of HRV, which was purified by using CsCl and treated with Triton X-100 under the different incubation time. 1; 0min, 2; 5min, 3; 10min, 4; 20min, 5; 40min, 6; 60min

## DISCUSSION

The SDS-PAGE patterns formed by the proteins of fish lyssaviruses HRV, IHNV, and VHSV were similar to each other, but clearly different from that of the vesiculoviruses PFR, SVCV, EVEX, and EVA. However, HRV, IHNV, and VHSV could be distinguished from each other by the molecular weights of the G, N, M1, and M2 proteins. Hsu et al. (1986) examined 71 isolates of IHNV from North America by SDS-PAGE and described 5 electropherotypes based upon differences in relative mobilities of the G and N proteins. In our SDS-PAGE gels, the estimated molecular weight of the N protein of HRV fell outside of the range of molecular weights reported for those strains of IHNV. Yoshimizu et al. (1987) showed that 11 isolates of HRV in Japan had the same SDS-PAGE patterns.

Two-dimensional gel electrophoresis was used to compare the structural proteins of HRV, IHNV, and VHSV. The pIs of M1 and M2 proteins of HRV were more acidic than those of IHNV or VHSV and could be used to distinguish HRV from IHNV or VHSV. The M1 protein of both HRV and IHNV formed 2 spots with different pI values. This may be due to the differences in the degree of phosphorylation of the M1 protein of these viruses. McAllister & Wagner (1975) determined both the N and M1 proteins of IHNV, but only N protein of VHSV was a phosphoprotein.

Western blot analysis revealed significant antigenic relatedness between the G, N, and M2 proteins of HRV, IHNV, and VHSV. While the extent of these cross-reactions can be expected to vary depending upon the source of the antiserum, we did not detect any cross-reaction between the M1 proteins of HRV and IHNV suggesting they are unique. While the structural proteins of the fish lyssaviruses appeared to share a large number of antigenic determinants, there was no antigenic relatedness with structural proteins of the fish vesiculoviruses indicating a greater evolutionary distance. Our results are analogous to those reported by Jorgensen et al. (1989) who performed a western blot analysis of the structural proteins of SVCV, PFR, and VHSV using antisera against SVCV and PFR. These antisera reacted specifically with the Ns protein of the homologous virus, but showed cross-reactions between the G and N protein of SVCV and PFR. In addition, the PFR antiserum reacted with the M proteins of both viruses. No cross-reaction was seen with any of the VHSV structural proteins.

Triton X-100 treatment of the HRV and IHNV virions showed that the G protein of IHNV was easily removed by treatment with Triton X-100 in low ionic strength buffer. In the presence of Triton X-100 and salt, the M1 and M2 proteins of IHNV were solubilized in addition to the G protein. These results are consistent with the findings of McAllister & Wagner (1975) and Lenoir & de Kinkelin (1975). In contrast, the structural proteins of HRV were substantially more stable in the presence of Triton X-100 or Triton X-100 containing NaCl. It is not clear why the virions of HRV were more stable than those of IHNV.

When HRV and IHNV purified by CsCl were treated with Triton X-100, a decrease in the amount of the M1 protein and the appearance of a new protein were observed, however, the molecular weight of the new protein was quite different between the two viruses. It seems likely that the newly appearing protein was produced by the processing of the M1 protein because it was not observed among the viral proteins synthesized by cells infected with fish lyssaviruses (Nishizawa et al. 1991c). This newly appearing protein could also be used to distinguish HRV from IHNV. White (1990) reported a similar reaction among orthomyxoviruses where the hemagglutinin (HA) protein is divided into HA1 and HA2 by processing to

become activated as a fusion peptide.

In this study, it was found that HRV, IHNV, and VHSV could be distinguished by differences in relative mobility of G, N, M1, and M2 proteins, lack of antigenic relatedness of the M1 protein, differing pI values of the M1 and M2 proteins, stability of G, M1, and M2 proteins in the presence of Triton X-100, and differences in the molecular weight of a new protein produced by processing of the M1 protein. These results also suggest that the M1 protein of fish lyssaviruses has a potential role in the recognition of virus by cells or in the ability of virus to infect and replicate within these cells.

It remains for future work to elucidate the role of M1 protein of fish lyssaviruses.

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