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Characteristics of three rhabdoviruses from snakehead fish *Ophicephalus striatus*

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ABSTRACT: Protein profiles and serological characteristics of 3 rhabdoviruses from snakehead fish *Ophicephalus striatus* were determined and compared to 5 known fish rhabdoviruses and 1 mammalian rhabdovirus. The snakehead rhabdovirus (SHRV) exhibited a bacilliform morphology and a Lyssavirus-type protein profile. The ulcerative disease rhabdovirus isolates (UDRV-BP and UDRV-19) were indistinguishable and exhibited bullet-shaped morphology and a Vesiculovirus-type protein profile. At present, none of the 3 viruses is known to be the cause of disease in any species of fish. UDRV-BP and UDRV-19 were serologically identical but distinct from SHRV and from 5 other fish rhabdoviruses. SHRV was serologically unrelated to any of the fish rhabdoviruses examined.

INTRODUCTION

Since 1980, a severe epizootic disease, often characterized by necrotic ulcerations, has occurred among both wild and cultured snakehead fish *Ophicephalus striatus* in southeast Asia, Malaysia, Thailand, Lao People's Democratic Republic and Burma (Boonyaratpalin 1989).

Various organisms (viruses, bacteria, fungi and higher parasites) were found to be associated with the diseased fish (Hedrick et al. 1986, Tonguthai 1986, Wattanavijarn et al. 1986, Boonyaratpalin 1989, Frerichs et al. 1989). Six rhabdovirus isolations have been reported from snakehead fish with ulcerative disease; however, there is no known causal relationship. The snakehead rhabdovirus (SHRV) was isolated in Thailand by Wattanavijarn et al. (1986). Five isolations of ulcerative disease rhabdovirus (UDRV), 3 in Thailand and 2 in Burma, were made by Frerichs et al. (1989), and all were serologically homologous. Although the viruses have been individually described (Ahne et al. 1988), no detailed comparative studies have been conducted. In this report, the morphology, structural proteins and serological characteristics of 3 isolates (SHRV, UDRV-BP and UDRV-19) are described and compared to each other and to other rhabdoviruses.

MATERIALS AND METHODS

Viruses and cell lines. SHRV was obtained in Thailand by Wattanavijarn et al. (1986) from snakehead with ulcerative disease; UDRV-BP and UDRV-19 were isolated from pooled organs of diseased fish in Thailand and Burma, respectively, by Frerichs et al. (1986). Five other fish rhabdoviruses, infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), hirame rhabdovirus (HRV), spring viremia of carp virus (SVCV) and pike fry rhabdovirus (PFRV), were used for comparison. Vesicular stomatitis virus (VSV) New Jersey serotype, a mammalian rhabdovirus, was used for protein profile comparison.

All fish rhabdoviruses, except UDRV-BP and UDRV-19, were propagated and titered in the *Epithelioma*

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papulosum cyprini (EPC) cell line (Fijan et al. 1983) cultured in Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 5 % fetal bovine serum (FBS). VSV was propagated in baby hamster kidney cells (BHK-21) in MEM. UDRV-BP and UDRV-19 were grown in the snakehead fin cell line (SHF) (Kasornchandra et al. 1988) in Leibovitz's L-15 medium supplemented with 5 % FBS. All culture media contained 100 U penicillin and 100 mg streptomycin ml^{-1}. The viral titer ($TCID_{50}$ ml^{-1}) was determined by end-point dilution assay using 96-well plates with 6 wells per dilution and calculated by the method of Reed & Muench (1938). Cells were incubated for 7 d at 27 °C.

Electron microscopy. Both SHRV and UDRV were inoculated on monolayer cell cultures at a multiplicity of infection (MOI) of 0.1, and incubated at 27 °C for 10 h. The culture medium was then decanted and the cell sheet washed 3 times with Hanks' balanced salt solution (HBSS, pH 7.4) and fixed for 2 h with 2.5 % glutaraldehyde in HBSS. The cell sheet was then washed 3 times with 0.2M cacodylate buffer (pH 7.3), harvested with a scraper, and centrifuged at 1000 × *g* for 10 min. The pellet was post-fixed with 1 % osmium tetroxide in 0.2M sodium cacodylate buffer for 1 h, washed, dehydrated, and embedded in Medcast-Aradite 502. The cells were sectioned and viewed with a Zeiss EM10/A at 60 kV.

Virus purification. All viruses were purified in the following manner. The cell monolayers were inoculated at an MOI of 0.001 and incubated at appropriate temperatures (Wolf 1988) until cytopathic effect (CPE) was complete. Culture fluid was harvested, clarified by centrifugation at 4000 × *g* for 10 min, and the virus concentrated by centrifugation at 80 000 × *g* for 90 min. The viral pellet was resuspended in 0.01M tris-HCl buffer, pH 7.5, and purified on discontinuous and continuous sucrose gradients (Engelking & Leong 1989). Purified virus was resuspended in 0.35 ml tris-HCl buffer and stored at -70 °C.

Analysis of viral structural proteins. Viral proteins denatured with SDS were separated by discontinuous polyacrylamide gel electrophoresis (SDS-PAGE). Using a 4.75 % stacking and 10 % separating gel (Laemmli 1970), polypeptides were electrophoresed under a constant 200 V for 45 min and visualized using a silver nitrate stain.

Glycosylation of viral glycoproteins. The glycosylated proteins of purified SHRV, UDRV-BP and UDRV-19 were identified by enzyme-linked immunosorbant assay (ELISA) using a glycan detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). Transferrin was used as a positive control and the standard molecular weight protein markers served as a negative control.

Polyclonal antibody production. Polyclonal mouse antibody to SHRV, HRV, SVCV, PFRV, UDRV-BP and UDRV-19 was prepared as follows. Viral protein (60 µg ml^{-1}) was mixed with an equal amount of Freund's complete adjuvant and the emulsion was injected intraperitoneally (IP) into three 8-wk-old female BALB/c mice. Two booster injections containing 30 µg of viral protein mixed with Freund's incomplete adjuvant (Sigma) were given IP at 1 mo intervals. Four days after the second booster, the mice were primed with an IP injection of 1.0 mg viral protein. Four days later, 3.3×10^6 sarcoma 180/TG cells in 0.3 ml sterile saline (0.15M NaCl) were injected IP. Ascitic fluid was collected after 10 to 15 d, clarified by centrifugation at 1000 × *g* for 5 min, passed through a 0.45 µm sterile membrane filter, and stored in 1.0 ml aliquots at -70 °C.

Cross-neutralization test. Cross-neutralization was performed by the alpha method of Rovozzo & Burke (1973). Each 10-fold virus dilution was reacted at 22 °C for 1 h with an equal volume of titered polyclonal antibody diluted appropriately for neutralization of 100 $TCID_{50}$'s of homologous virus. The virus-antibody combination was assayed for infectivity by inoculating 0.2 ml of each dilution into each of 3 wells of a microplate containing a confluent monolayer. IHNV, VHSV and HRV infected cultures were incubated at 14 °C; SVCV and PFRV at 20 °C; and SHRV, UDRV-BP and UDRV-19 at 27 °C. Incubation was continued until obvious CPE had developed in the virus-control wells and the log_{10} neutralization indices (NI) were determined (Rovozzo & Burke 1973).

RESULTS

Electron microscopy

SHRV had bacilliform morphology (Fig. 1a), with a range of 180 to 200 nm in length and 60 to 70 nm in width. In contrast, UDRV-BP and UDRV-19 particles exhibited a bullet-shape morphology (Fig. 1b, c) with size ranging from 110 to 130 nm in length and 50 to 65 nm in width. Numerous virus particles were found in the cytoplasm and budding through the cell membranes.

Analysis of viral structural proteins

The polypeptides of the 9 rhabdoviruses separated by SDS-PAGE gave 2 distinct patterns (Fig. 2). The first pattern, exhibited by IHNV, VHSV, HRV and SHRV, was similar to that of rabies virus (Lenoir & de Kinkelin 1975, Coslett et al. 1980). The second pattern, exhibited by SVCV, PFRV, UDRV-BP and UDRV-19, more closely resembled that of VSV as seen in Lane 9 (McAllister & Wagner 1975, Wagner 1975).

The estimated molecular weights of the structural proteins of SHRV, IHNV, VHSV and HRV are shown in Table 1. Although the banding patterns were similar for these 4 viruses, differences in the migration of individual proteins were observed. These differences are reflected in the range of molecular weights determined for the G, N, M1 and M2 proteins.

The molecular weights of the viral proteins of SVCV, PFRV, UDRV-BP and UDRV-19 were estimated (Table 2). The proteins of the 2 UDRV isolates were equivalent in size. Though evident for UDRV-BP and UDRV-19, the non-structural (NS) proteins of SVCV, PFRV and VSV could not be identified (Fig. 2), but the N and M proteins of the UDRV isolates were intermediate in size between those of SVCV and PFRV, and the UDRV G protein was smaller than either of the other two. It was determined, by ELISA, that the 68 kDa protein of SHRV and the 71 kDa protein of UDRV-BP and UDRV-19 were glycosylated.

Cross-neutralization tests

The greatest neutralizing activity in the mouse ascitic fluid was obtained with SVCV with a titer of 1:126, and the lowest titer was from HRV at 1:64. The mouse ascitic fluid had a toxic effect on the cells, so the lowest dilution permitting detection of viral CPE was 1:16. The anti-IHNV and anti-VHSV rabbit antisera had neutralizing titers of 1:100 and 1:256, respectively. In the cross-neutralization assay, 8 fish rhabdoviruses were compared (Table 3). SHRV, IHNV, VHSV, HRV, SVCV and PFRV were not significantly neutralized by antisera to any of the heterologous viruses. However, strong cross-neutralization occurred between the UDRV-BP and UDRV-19.

DISCUSSION

Six rhabdovirus isolations have been made from the diseased snakehead fish in Thailand and Burma. Characteristics of 3 rhabdovirus isolates, SHRV and UDRV-BP from Thailand and UDRV-19

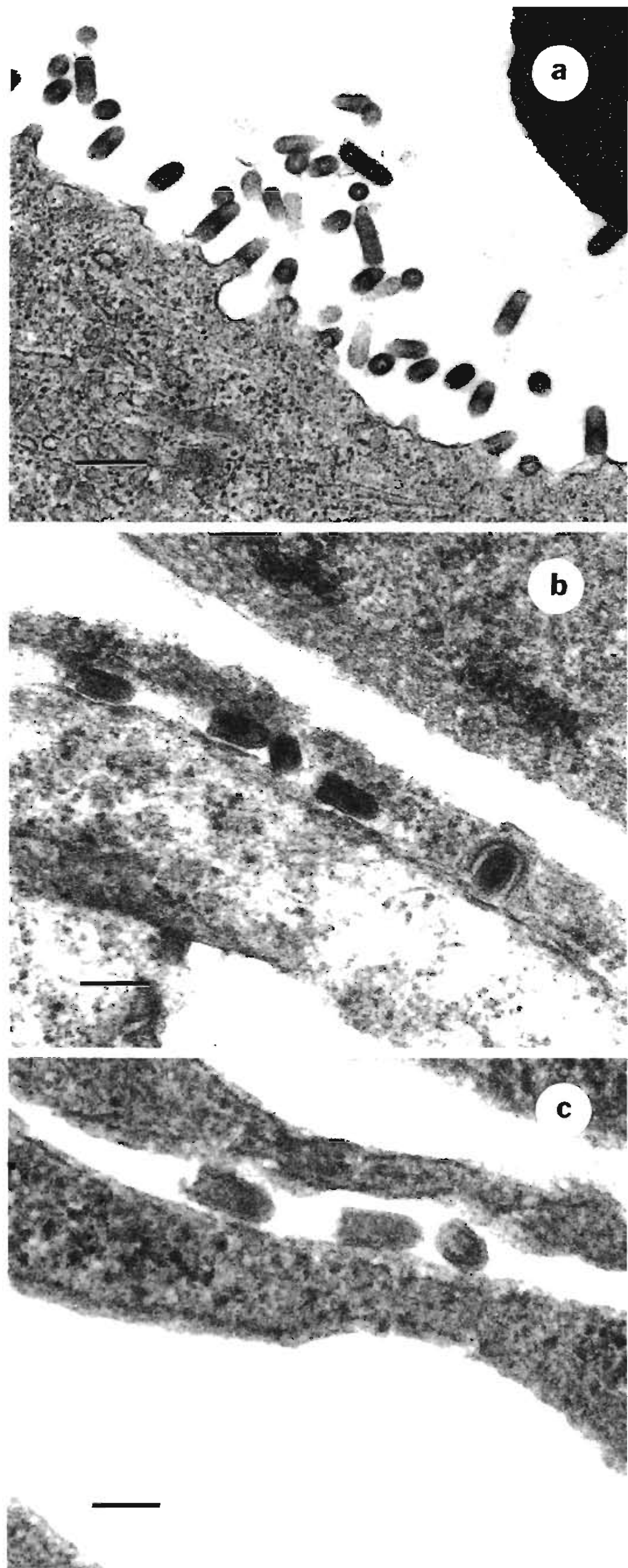

Fig. 1. (a) Electron micrograph of an ultrathin section of EPC cells infected with the snakehead rhabdovirus (SHRV). Scale bar = 260 nm. (b) Thin section of SHF cells infected with the ulcerative disease rhabdovirus (UDRV-BP). Scale bar = 143 nm. (c) Thin section of SHF cells infected with the ulcerative disease rhabdovirus (UDRV-19). Scale bar = 97 nm

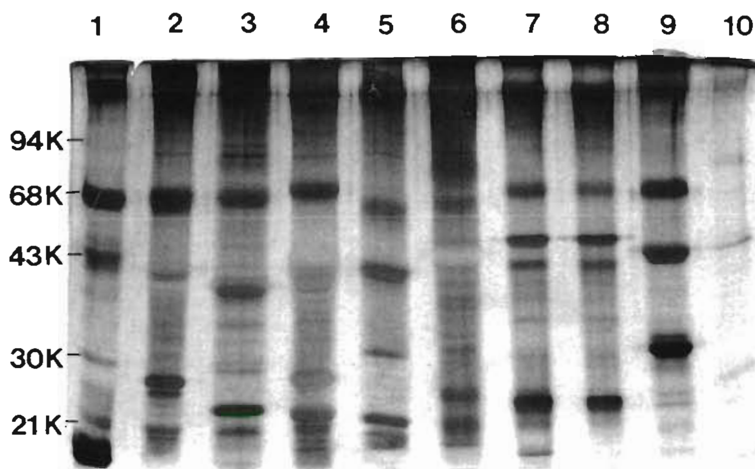

Fig. 2. A comparison of the viral polypeptides of 3 snakehead rhabdovirus isolates, 5 other fish rhabdoviruses and a mammalian virus, VSV, by polyacrylamide gel electrophoresis. The gel was stained with silver nitrate. Lane 1: molecular weight markers phosphorylase B (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). Lane 2: snakehead rhabdovirus (SHRV). The estimated molecular weight of the 5 virion protein components of SHRV were >150, 68, 42, 26.5 and 20 kDa. These proteins likely correspond to those previously characterized as the L, G, N, M1 and M2 structural proteins of IHNV, VHSV and HRV (McAllister & Wagner 1975, Hsu et al. 1985, Kimura et al. 1989). Lane 3: infectious hematopoietic necrosis virus (IHNV). Lane 4: viral hemorrhagic septicemia virus (VHSV). Lane 5: hirame rhabdovirus (HRV). Lane 6: spring virema of carp virus (SVCV). Lane 7: ulcerative disease rhabdovirus (UDRV-BP). Lane 8: ulcerative disease rhabdovirus (UDRV-19). These may correspond to the L, G, N and M proteins of SVCV and PFRV (Lenoir 1973, de Kinkelin et al. 1974). A minor protein of both UDRV-BP and UDRV-19 with a molecular weight of 48 kDa was also observed in this gel. Lane 9: vesicular stomatitis virus (VSV). Lane 10: pike fry rhabdovirus (PFRV)

from Burma, were described and compared. Electron micrographs of these viruses in thin section showed that the particle size of UDRV-BP and UDRV-19 was the same, and both had a typical bullet shape similar to that of other fish rhabdoviruses (Hill et al. 1975, 1980, Kimura et al. 1986). Our observations of the size and shape of the UDRV isolates were similar to those reported by Frerichs et al. (1986) and were different from those of the SHRV. In contrast to the UDRV isolates, SHRV exhibited bacilliform morphology that is commonly found in plant rhabdoviruses (Hetrick 1989), although Malsberger & Lautenslager (1980) reported a rhabdovirus (Rio Grande perch rhabdovirus) with bacilliform morphology that was isolated from a fish of the family Cichlidae.

The 8 fish rhabdoviruses tested (IHNV, SHRV, VHSV, HRV, SVCV, UDRV-BP, UDRV-19 and PFRV) can be separated into 2 groups based on their SDS-PAGE protein profiles. The first group (IHNV, SHRV, VHSV, HRV) was composed of virions whose structural protein profile closely resembled that of rabies virus, the prototype virus of the *Lyssavirus* genus of the family Rhabdoviridae. These proteins are classified as L for the polymerase, G for the surface glycoprotein, N for the nucleocapsid and M1 and M2 for the envelope matrix proteins (Wagner et al. 1972, Lenoir & de Kinkelin 1975, McAllister & Wagner 1975). The second group of viruses contained SVCV, UDRV-BP, UDRV-19 and PFRV. These viruses are composed of 4 major structural proteins (L, G, N and M). In addition, both UDRV-BP and UDRV-19 showed a minor structural protein with a molecular weight of 48 kDa similar to the structural protein (NS) of VSV. A minor protein of similar molecular weight has been reported for SVCV and PFRV but was not detected by us (Wolf 1988). Based on structural protein profile and molecular weights, UDRV-BP and UDRV-19 seem identical. The protein banding patterns of these viruses resemble that of VSV, the prototype virus of the genus *Vesiculovirus* (Wagner 1975).

By ELISA, the presence of a single glycosylated structural protein of SHRV with an apparent molecular weight of 68 kDa was detected. Similarly, UDRV has a single structural glycosylated protein of the somewhat greater apparent molecular weight of 71 kDa (Fig. 2). The rhabdoviral glycoprotein

Table 1 Estimated molecular weights (× 10^3 Da) of virion structural proteins of snakehead rhabdovirus (SHRV), infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia (VHSV) and hirame rhabdovirus (HRV)

Lyssavirus-like group					
Virus	Polymerase L	Glycoprotein G	Nucleoprotein N	Matrix proteins M1	M2
SHRV	>150	68	42	26.5	20
IHNV	>150	67	40.5	28	22.5
VHSV	>150	72	42	26.5	22
HRV	>150	60	42.5	30	22

Table 2. Estimated molecular weights ($\times 10^3$ Da) of virion structural proteins of spring viremia of carp virus (SVCV), ulcerative disease rhabdoviruses (UDRV-BP and UDRV-19) and pike fry rhabdovirus (PFRV)

Vesiculovirus-like group				
Virus	Polymerase L	Glycoprotein G	Nucleoprotein N	Matrix protein M
SVCV	>150	85	45	23
UDRV-BP	>150	71	53	22
UDRV-19	>150	71	53	22
PFRV	>150	80	43	24

Table 3. Cross-neutralization tests for 8 fish rhabdoviruses

Virus	Polyclonal antisera							
	SHRV 1:112[a]	IHNV 1 100	VHSV 1:256	HRV 1:64	SVCV 1:126	UDRV-BP 1.64	UDRV-19 1:64	PFRV 1.96
Snakehead rhabdovirus (SHRV)	2.0[b]	0	0	0	0	0	0	0
Infectious hematopoietic necrosis virus (IHNV)	0	1.8	0	0	0	0	0	0
Viral hemorrhagic septicemia virus (VHSV)	0	0	1.8	0	0	0	0	0
Hirame rhabdovirus (HRV)	0	0	0	1.8	0	0	0	0
Spring viremia of carp virus (SVCV)	0	0	0	0	2.0	0	0	0
Ulcerative disease rhabdovirus (UDRV-BP)	0	0	0	0	0	2.0	1.9	0
Ulcerative disease rhabdovirus (UDRV-19)	0	0	0	0	0	1.9	2.0	0
Pike fry rhabdovirus (PFRV)	0	0	0	0	0	0	0	1.8

[a] The neutralizing titer against 100 $TCID_{50}$ of homologous virus
[b] Log of neutralization index (Rovozzo & Burke 1973)

occurs on the viral envelope and can elicit neutralizing antibody (Kelley et al. 1972, Cox et al. 1977, Engelking & Leong 1989). By western blot, the polyclonal antibody against SHRV reacted with the G protein of all the fish rhabdoviruses examined. A similar result was obtained with polyclonal antiserum to the purified G protein of IHNV when reacted with SHRV, IHNV and VHSV (data not shown). This cross-reaction may indicate a conserved, non-neutralizing, linear epitope in the G protein of these fish rhabdoviruses. This region may possibly be important in anchoring the G protein to the capsid.

Serological comparison of SHRV, UDRV-BP, UDRV-19 and 5 other fish rhabdoviruses showed that SHRV is unique and that UDRV-BP and UDRV-19 are serologically identical but distinct from other fish rhabdoviruses examined. Although the SHRV and UDRV were originally obtained from the same host species, they can be distinguished by their morphology, serological characteristics and structural proteins. Both rhabdoviruses from snakehead fish should be regarded as distinct viruses. The relation of these viruses to the ulcerative disease of snakehead fish is unknown.

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