Characterization of a virus obtained from snakeheads *Ophicephalus striatus* with epizootic ulcerative syndrome (EUS) in the Philippines

Gilda D. Lio-Po[1,*], Garth S. Traxler[2], Lawrence J. Albright[3], Eduardo M. Leaño[1]

[1]Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo 5021, Philippines
[2]Pacific Biological Station, Nanaimo, British Columbia, V9R 5K6, Canada
[3]Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, V5A 1S6, Canada

ABSTRACT: This is the first report of the isolation and characterization of a fish virus from the Philippines. The virus was isolated using snakehead spleen cells (SHS) from severely lesioned epizootic ulcerative syndrome (EUS)-affected snakehead *Ophicephalus striatus* from Laguna de Bay, in January 1991. The virus induced cytopathic effects (CPE) in SHS cells yielding a titer of 3.02×10^6 TCID$_{50}$ ml^{-1} at 25°C within 2 to 3 d. Other susceptible cell lines included bluegill fry (BF-2), catfish spleen (CFS) and channel catfish ovary (CCO) cells. Replication in chinook salmon embryo cells (CHSE-214) was minimal while *Epithelioma papulosum cyprini* cells (EPC) and rainbow trout gonad cells (RTG-2) were refractory. Temperatures of 15 to 25°C were optimum for virus replication but the virus did not replicate at 37°C. The virus can be stored at –10 and 8°C for 30 and 10 d, respectively, without significant loss of infectivity. Viral replication was logarithmic with a 2 h lag phase; viral assembly in the host cells occurred in 4 h and release of virus occurred 8 h after viral infection. A 1-log difference in TCID$_{50}$ titer between the cell-free virus and the total virus was noted. Freezing and thawing the virus caused a half-log drop in titer. Viral exposure to chloroform or heating to 56°C for 30 min inactivated the virus. Exposure to pH 3 medium for 30 min resulted in a more than 100-fold loss of viral infectivity. The 5-iododeoxyuridine (IUdR) did not affect virus replication, indicating a RNA genome. Neutralization tests using the Philippine virus, the ulcerative disease rhabdovirus (UDRV) and the infectious hematopoietic necrosis virus (IHNV) polyvalent antisera showed slight cross-reaction between the Philippine virus antiserum and UDRV but established no serological relationship with SHRV and IHN virus. Transmission electron microscopy (TEM) of SHS cells infected with the virus showed virus particles with typical bullet morphology and an estimated size of 65×175 nm. The Philippine virus was therefore a rhabdovirus, but the present study did not establish its role in the epizootiology of EUS.

KEY WORDS: Epizootic ulcerative syndrome (EUS) · Fish rhabdovirus · Characterization · *Ophicephalus striatus* · Snakehead · Disease · Cell susceptibility

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is a malady among wild and cultured freshwater fishes in the Philippines and in many Asian countries. Lesions consist of severe ulcerative dermal necrosis with extensive erosion/sloughing of the underlying musculature and are often associated with a foul odor (Lio-Po et al. 1992). Fish species severely affected are snakeheads *Ophicephalus striatus*, catfish *Clarias batrachus*, gourami *Trichogaster trichopterus* and *T. pectoralis*, goby *Glossogobius guirus*, silvery theraponid *Therapon plumbius*, climbing perch *Anabas testudineus*, barbs *Pun-*

*E-mail: liopo@aqd.seafdec.org.ph

tius spp., serpent fish *Channa* spp. and spiny eels *Fluta alba* (Tonguthai 1985). Outbreaks are more common between the months of September and March, which correlates with the time when lowest water temperatures occur.

Several species of bacteria and fungi have been found to be associated with EUS-affected snakeheads *Ophicephalus striatus*, but these have not been conclusively shown to be the primary disease agents of EUS (Lio-Po et al. 1992, 1996, 1998, Roberts et al. 1993, Callinan et al. 1995). Lilley & Roberts (1997) reported the histological invasiveness of the EUS-associated *Aphanomyces* through the fish muscles after experimental exposure, but the development of gross EUS-like lesions was not mentioned. Conversely, rhabdoviruses and a birnavirus associated with EUS-lesioned fish have been isolated in Thailand (Frerichs et al. 1986, Hedrick et al. 1986, Kasornchandra et al. 1991), but their virulence has also not been established (Frerichs et al. 1993).

This study reports on the characteristics of a virus isolate, 91-97, isolated from an EUS-affected snakehead collected from Laguna de Bay, Philippines, in January 1991.

MATERIALS AND METHODS

Cell cultures. Atlantic salmon (AS), bluegill fry (BF-2) (Wolf & Quimby 1966), channel catfish ovary (CCO) (Bowser & Plumb 1980), chinook salmon embryo (CHSE-214), *Epithelioma papulosum cyprini* (EPC) (Fijan et al. 1983), rainbow trout gonad (RTG-2), catfish spleen (CFS) and snakehead spleen (SHS) (Lio-Po et al. 1999) cells were used. The CCO cells provided by RP Hedrick (University of California-Davis, USA) and the other cells provided by G.S.T. were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS) (MEM-10). SHS and CFS were maintained in Leibovitz medium (L15) supplemented with 10% FBS (L15-10).

Virus isolation. The test virus, 91-97, was isolated from several virus-positive filtrates obtained from severely lesioned EUS-affected snakeheads (mean weight = 175 g) from Laguna de Bay, Philippines, in January 1991. External lesions and visceral organs (pool of spleen, liver, kidney and gills) were aseptically excised and transported in L15 medium containing 200 IU penicillin ml^{-1} and 200 µg streptomycin ml^{-1}. Tissue homogenates were prepared, diluted to 10% (Chong et al. 1990) with Earle's balanced salt solution (EBSS) containing antibiotics and 50 µg amphotericin B ml^{-1} (antibiotic-antimycotic mixture A). The homogenates were then centrifuged at 3000 × *g* for 15 min at 4°C. The supernatant was filtered through a 0.45 µm

pore size membrane filter (Millipore) and stored at –70°C until virus assay.

Primary viral isolation was conducted in SHS cells in 24-well plates (Falcon) with L15 medium containing 4% FBS (L15-4) buffered with 1M N-2-hydroxyethyl-piperazine-N′-2ethanesulfonic acid (Hepes). The plates were then incubated at 25°C for 7 d. Blind passages were carried out on negative samples at least 3 times. Only isolates manifesting consistent cytopathic effects (CPE) in all subsequent passages were considered positive. The pH of all media used was adjusted to 7.2–7.4 with 7.5% sodium bicarbonate and supplemented with 100 IU penicillin G sodium, 100 µg streptomycin sulfate, 25 µg amphotericin B ml^{-1} medium.

Other viruses. The snakehead rhabdovirus (SHRV) was provided by J. L. Fryer and J. Kasornchandra (Oregon State University, USA) while the infectious hematopoietic necrosis virus (IHNV) came from G.S.T. Ulcerative disease rhabdovirus (UDRV) was provided by G. N. Frerichs (Stirling University, UK).

Virus propagation and assay. The 91-97 viral isolate and the SHRV were propagated in SHS cells at 25°C for 2 to 5 d while IHNV was grown in CHSE-214 cells at 15°C for 2 wk. All viral titers were determined by the end-point dilution assay (TCID$_{50}$) of Rovozzo & Burke (1973).

Susceptibility of cell lines. The susceptibility of different cell lines to the 91-97 virus isolate was tested using BF-2, CFS, CCO, EPC, and SHS at 25°C for 5 d and AS, CHSE-214, RTG-2 at 20°C for 7 d.

Virus replication at different incubation temperatures. Confluent SHS cells in 25 cm tissue culture flasks (Falcon) were inoculated at a multiplicity of infection (MOI) of 0.001 with the 91-97 virus. Seven inoculated flasks were incubated at each of the following temperatures: 15, 20, 25, 30 and 37°C. Each day for 7 d, 1 flask incubated at each test temperature was removed and the contents frozen at –70°C. After the experiment these fluids were rapidly thawed, clarified by low speed centrifugation, and the virus titer of each determined.

Virus stability at varying temperatures. Clarified viral suspensions were dispensed in 10 ml aliquots in sterile tubes and stored at various temperatures: –10, 4, 20 and 30°C. After 3, 6, 10, 30, 60 and 120 d, the virus titers were assayed.

Plaque assay. Plaque assay of the 91-97 virus was conducted in 8 well plates (Nunc) with SHS cells following the method of Burke & Mulcahy (1980). At termination, the monolayers were overlaid with a formalin fixative-crystal violet stain solution and the plaques estimated as plaque forming units (pfu) ml^{-1}.

Growth curve determination. The virus was inoculated at an MOI of 0.001 onto SHS cells grown in 25 cm flasks and incubated at 25°C. At 2 h intervals for

24 h, 4 flasks were removed and the titer of free and total virus assayed. Cell-free virus titer was determined from the supernatant pool of 2 flasks following low speed centrifugation. Total virus was determined from a pool of 2 flasks of both supernatant and homogenized cells after centrifugation. Both preparations were frozen at −70°C, then titered at the same time in 96-well plates at the end of the 24 h experiment.

Lipid solvent sensitivity. One ml clarified supernate containing the test virus was mixed with 0.5 ml chloroform (reagent grade) or L15 medium alone according to the method of Feldman & Wang (1961). After 10 min shaking at room temperature, the tubes were centrifuged at $1000 \times g$ for 10 min and the uppermost clear layer was removed for viral titration.

Sensitivity to 5-iododeoxyuridine (IUdR). Ten-fold serial dilution of the virus was inoculated on monolayers of SHS cells pretreated with $10^{−4}$ M IUdR (Sigma) in EBSS or EBSS alone according to the method of Rovozzo & Burke (1973). After 3 h incubation, the cells were washed and overlaid with L15-4 buffered with Hepes. The virus titers in both treatments were compared after 4 d incubation at 25°C.

Effect of heat. Aliquots of the virus were heated in a water bath to 56°C (Rovozzo & Burke 1973). After 0.5, 1, 2, 4, 6 and 8 h incubation, the treated virus were each titered. Unheated virus inocula was used as control.

pH sensitivity. L15 medium adjusted to pH 3 or pH 7 was inoculated with the virus (Rovozzo & Burke 1973). After 30 min incubation at 25°C, the virus were titered. The pH 3 treatment was neutralized with 0.1 N NaOH before viral titration.

Stability to freeze-thaw. An aliquot of the virus was titered and frozen at −70°C. After each repeated freezing and thawing the virus titers were determined for up to 3 cycles according to Kasornchandra et al. (1991).

Purification of the virus. The virus was grown in SHS cells supplemented with 2% bovine serum albumin (BSA) (Sigma) (Wunner 1985) until complete destruction of the monolayer. After low speed centrifugation at 4°C, the supernatant was purified by sucrose gradient ultracentrifugation (Engelking & Leong 1989). The purified virus was resuspended in tris buffer and stored at −70°C.

Polyclonal antibody production. Approximately 100 μg of purified viral protein was mixed with incomplete Freund's adjuvant and injected subcutaneously into a New Zealand white female rabbit. After 2 wk, the rabbit was boosted with 50 to 90 μg of the antigen. Serum was collected after 2 wk, then stored in aliquots at −70°C. Normal rabbit serum was also collected before immunization as the negative control.

Neutralization test. Serological comparisons of the 91-97 virus with other warmwater fish rhabdoviruses associated with EUS (SHRV and UDRV) as well as IHNV were conducted by neutralization tests using polyclonal antisera raised against the 91-97 virus isolate, anti-IHNV from G.S.T. and anti-UDRV from G. N. Frerichs.

Serial 2-fold dilutions of each serum in Hanks' balanced salt solution (HBSS) were reacted with an equal volume of a 10^4 TCID$_{50}$ ml^{-1} of the 91-97 virus isolate for 1 h at 25°C. Thereafter, neutralization titers were estimated in SHS cells incubated at 25°C for 5 d based on the method of Rovozzo & Burke (1973). EPC cells were used for IHNV with MEM-4-hepes incubated at 15°C for 2 wk.

Electron microscopy. Snakehead spleen cells inoculated with the virus were fixed in 3% glutaraldehyde in Millonig's buffer and processed for resin blocking. The sections were stained with 1% (wt/vol) uranyl acetate and Reynold's lead citrate and viewed under the transmission electron microscope (TEM).

Experimental fishes. Cultured stocks of snakeheads are not available in the Philippines. Thus, healthy snakeheads, 40 to 80 g, from a river source in Iloilo, Philippines were used. The fish were acclimated at ambient temperature (27 to 32°C) for 1 wk. Since EUS outbreaks have never occurred in Iloilo and 10 fish samples were confirmed virus-free and without neutralizing antibodies, the test fish were considered to be naïve. At least 3 d prior to the infection experiments, the naïve fish were maintained at 20 to 25°C. Two fish were stocked per 5 l aquarium, each aquarium containing UV-sterilized unchlorinated freshwater.

Infection experiments and reisolation of the test virus. Healthy, naïve snakeheads were exposed to the virus by bathing dermally-abraded test fish or unabraided fish, or by injection using intraperitoneal (IP), intrakidney (IK), intraspleen (IS) and intradermal (ID) routes of administration. In a second experiment, snakeheads fed and unfed for 2 wk prior to the experiment were inoculated by intramuscular injection with the virus. In a third experiment, the test virus was tissue-passaged in healthy snakeheads 1 to 3 times before challenging naïve snakeheads by injection. Lesion development and mortalities were monitored daily for 2 wk. Lesion/muscle and visceral organ filtrates of experimental fish exposed to the virus were assayed for virus using SHS cells.

RESULTS

Infection of SHS cells with the virus induced CPE and yielded 3.02×10^6 TCID$_{50}$ ml^{-1} at 25°C within 2 to 3 d. Similarly, virus titer by plaque assay in SHS cells was 1.63×10^6 pfu ml^{-1}. Tests on various cell lines showed that BF-2, CFS, CCO and SHS cells were all

susceptible to the 91-97 virus isolate, producing similar virus titers of 10^6 $TCID_{50}$ ml^{-1}. The cytopathic effects consisted of granulation, rounding up and clumping, followed by detachment and lysis of cells (Fig. 1) among susceptible cell lines. Initial CPE was observed 24 h after viral inoculation, and there was complete destruction of the monolayer 2 to 3 d post-viral exposure. In CHSE-214, the virus titer was approximately 1.35×10^3 $TCID_{50}$ ml^{-1}, but when blind passaged in SHS, a titer of 10^7 $TCID_{50}$ ml^{-1} was attained. EPC, AS and RTG-2 cells were refractory to the test virus.

Fig. 2 shows the 91-97 virus replication at various incubation temperatures. Virus titers of at least 10^6 $TCID_{50}$ ml^{-1} were achieved at 25 and 30°C on Day 1 and at 15 and 20°C on Day 2 with continuous high titers until Day 6. At 15°C, viral replication was initially slower but virus titers as high as 2.13×10^7 $TCID_{50}$ ml^{-1} occurred on Days 4 to 7. There was no replication of the virus at 37°C.

Virus remained 'viable' for 30 d at –10°C. At 8°C, the virus remained stable for 10 d while at 20 and at 30°C, virus titer was drastically reduced after 3 d (Fig. 3).

Assay of the cell-free virus (S) and the total virus (C) showed that viral replication followed a typical pattern. Viral replication demonstrated a lag phase of 2 h with evidence of initial virus assembly in the host cells as early as 4 h after viral exposure. Release of the replicated virus from the host cells was detectable 8 h after infection. A 1-log difference in $TCID_{50}$ titer between the cell-free virus and the total virus was consistently observed thereafter until 12 h, indicating continuous replication and subsequent viral release.

The freeze-thaw cycle caused an approximate half-log drop in the titer of the 91-97 virus titer.

Table 1 summarizes the effect of several chemical and physical agents on the virus. Exposure to chloroform for 10 min or to heating to 56°C for 30 min inactivated the virus. Suspension at pH 3 for 30 min caused a loss of viral infectivity of more than 2 orders of magnitude. IUdR did not affect replication of the virus, indicating that it had an RNA genome. All negative controls yielded normal viral titers.

Neutralization tests showed that anti-UDRV antiserum cross-reacted with the 91-97 virus isolate (Table 2), but both anti-91-97 and anti-UDRV antisera
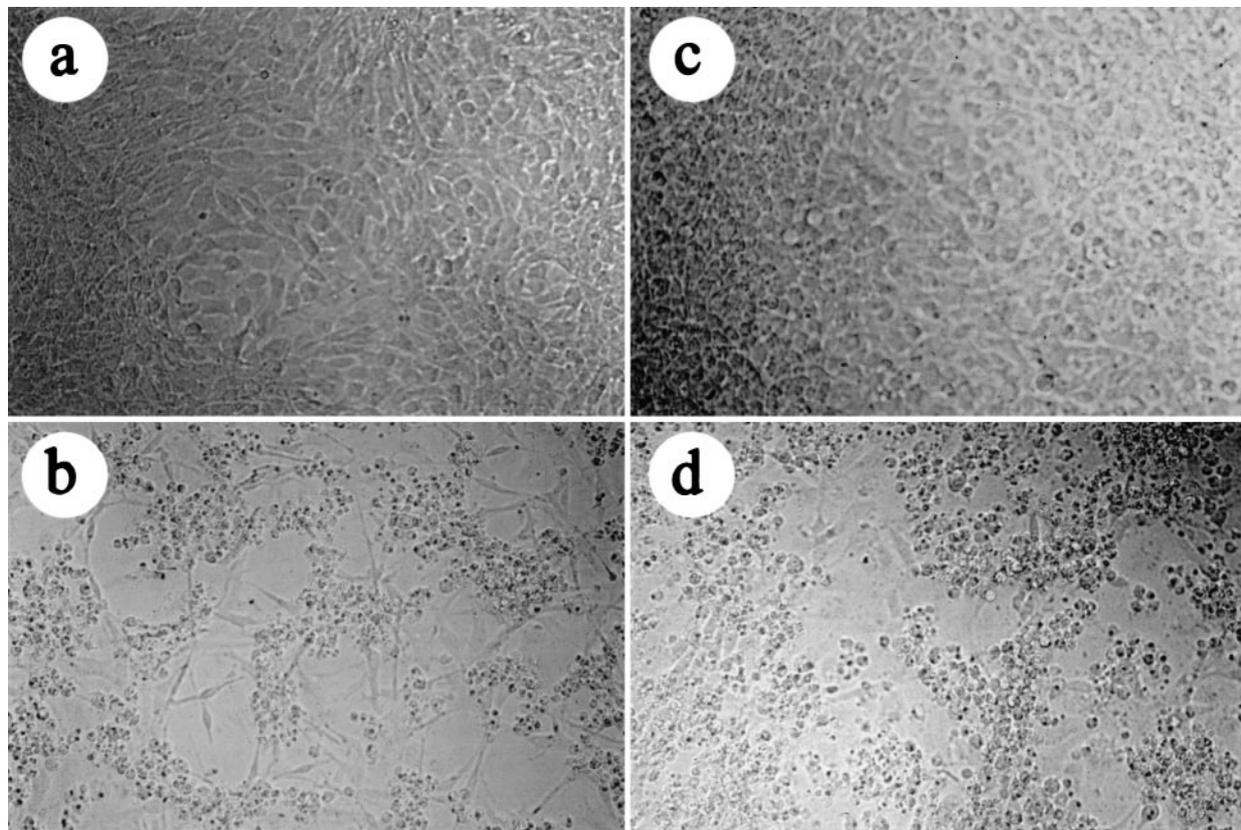

Fig. 1. (a) Monolayer of catfish spleen cells (CFS) cultured in Leibovitz medium and (b) CFS cells showing cytopathic effects due to the 91-97 virus isolate at 25°C; (c) monolayer of snakehead spleen cells (SHS) cultured in Leibovitz medium and (d) SHS cells with cytopathic effects caused by the 91-97 virus isolate at 25°C
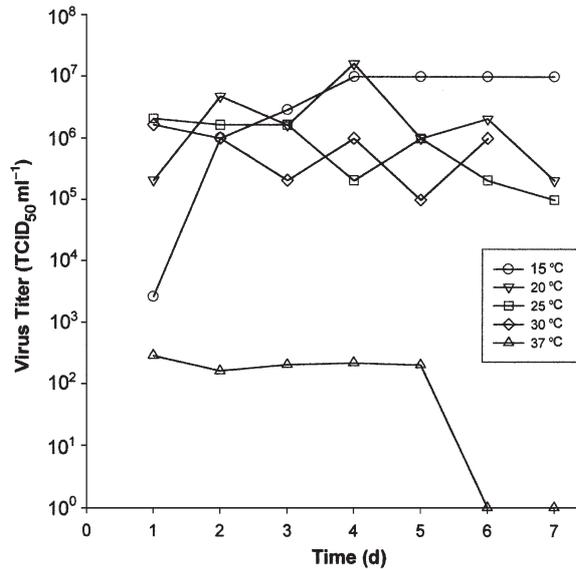
Fig. 2. Titers of the 91-97 virus isolate in snakehead spleen cells (SHS) following incubation in Leibovitz medium at different times (d) and temperatures (°C)

did not cross-react with SHRV. Anti-IHNV also did not react with the 91-97 virus isolate. The pre-immune serum of the rabbit from which the anti-91-97 virus was prepared yielded very high neutralization titers against UDRV that nullified the test for UDRV.

Fig. 4 illustrates the 91-97 virus isolate as viewed by TEM of SHS cells infected with the virus. The morphology of the virus is typical of a bullet-shaped rhabdovirus. The virus size is estimated as 65×175 nm.
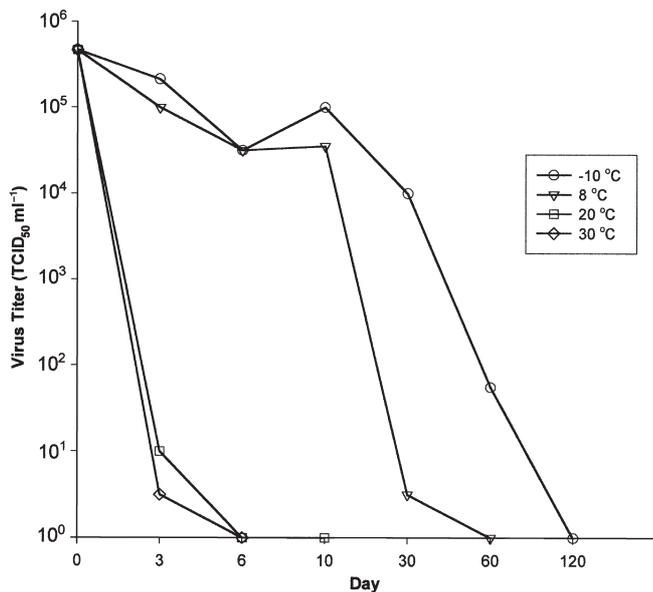

Fig. 3. Temporal stability of the 91-97 virus isolate at 4 temperatures (°C)

Table 1. Effect of several chemical agents and heat on the viability of the 91-97 virus isolated from EUS-affected snakehead *Ophicephalus striatus* and assayed in snakehead spleen cells at 25°C. IUdR: 5-iododeoxyuridine; EBSS: Earle's balanced salt solution

Agent	Inactivation
1:3 Chloroform (10 min)	Yes
Control:Leibovitz medium	No
Heat at 56°C (30 min)	Yes
Control: 25°C (30 min)	No
10^{-4} M IUdR (3 h)	No
Control:EBSS	No
pH 3 (30 min)	Yes
pH 7 (30 min)	No

No viral pathology was induced following experimental challenges. Virus, however, was recovered from visceral organs until Day 8 post-challenge for fish infected by the IP route and until Day 5 post-challenge in fish infected by intramuscular injection. The virus was detected predominantly in the gonad and kidney.

DISCUSSION

This is the first record of a virus isolated from a diseased fish in the Philippines.

The virus was sensitive to heat at 56°C, pH 3, and chloroform, which indicated the presence of a lipid-containing envelope. It was resistant to IUdR, a characteristic of viruses with RNA genomes. These attributes plus its typical bullet-shaped morphology identified it as a rhabdovirus.

The virus was propagated in a wide range of cells derived from fish species known to be susceptible to natural infections with viruses, such as CFS and SHS cells. Cells of BF-2 and CCO were also susceptible to

Table 2. Neutralization titers of 3 polyvalent antisera with the 91-97 virus isolate, UDRV, SHRV and IHNV. 91-97: virus isolated from EUS-lesioned snakehead in the Philippines; UDRV: ulcerative disease rhabdovirus; SHRV: snakehead rhabdovirus; IHNV: infectious hematopoietic necrosis virus; NT: not tested; *Results disregarded the cross-neutralization titer obtained because high titer was detected in the pre-immune serum

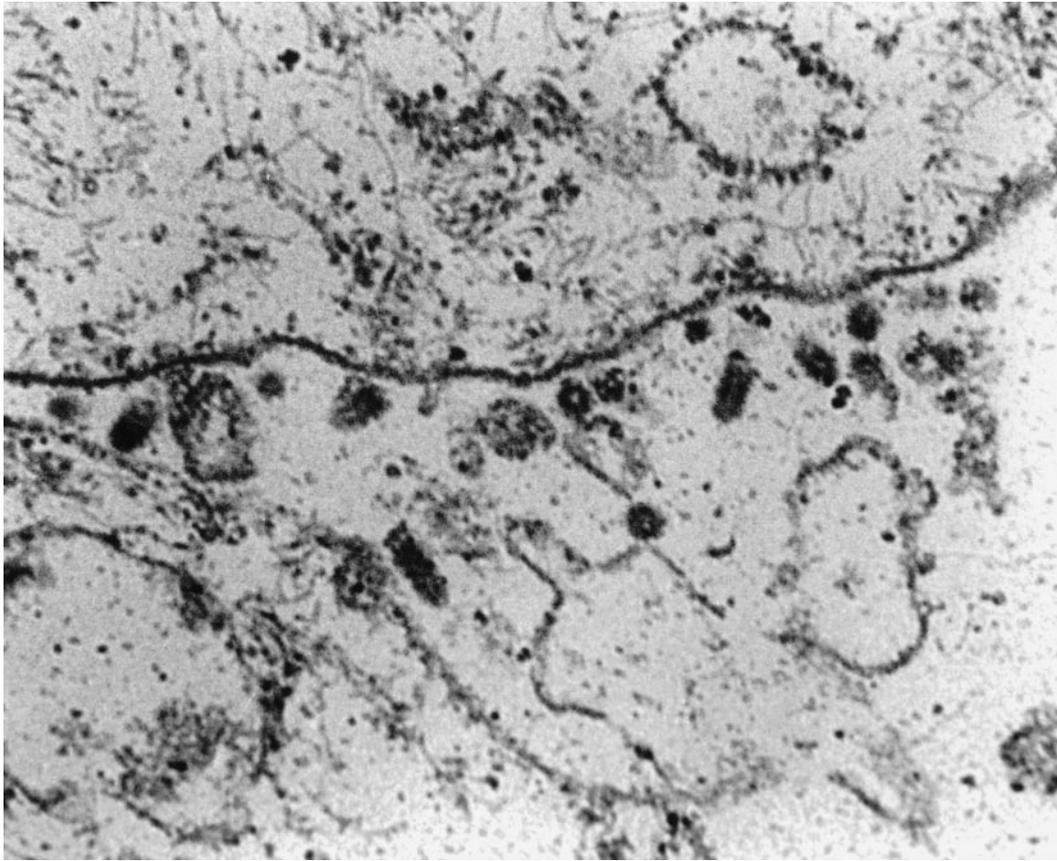| Virus	91-97 virus	Antisera UDRV	IHNV
91-97	91	25	0
UDRV	*	645	NT
SHRV	0	0	NT
IHNV	6	NT	6085

Fig. 4. Transmission electron photomicrograph of the 91-97 virus isolate after culture in snakehead spleen cells (SHS) for 72 h and stained with uranyl acetate and lead citrate (×77 000)

the virus. Growth in the CHSE-214 cells, however, was very minimal, while AS, EPC and RTG-2 were refractory to the test virus. Thus, the actual rate of virus maturation and release from infected cells was dependent on the type of cell line used.

The virus replicated rapidly in SHS cells. Incubation time from infection to initial assembly and release from cells was as fast as 4 and 8 h, respectively, which indicates a typical viral release pattern. This pattern is similar to that reported with the SHRV rhabdovirus isolated from EUS-affected fish in Thailand (Kasornchandra et al. 1991).

Optimum growth produced viral titers of 10^5 to 10^7 TCID$_{50}$ ml^{-1} over a wide temperature range of 15 to 30°C. While replication was most rapid at 25 and 30°C, the highest titer of 2.13×10^7 TCID$_{50}$ ml^{-1} was achieved at 15°C. The same observations were reported for UDRV (Frerichs et al. 1989). The replication temperature requirements corresponded with the water temperature ranges below 25°C occurring during the EUS outbreaks. In addition, the virus tolerated incubation at 37°C for 5 d but was inactivated by Day 6. These observations indicate that the 91-97 virus propagates

well at 15 to 30°C. However, 'viability' of the virus may be compromised at 30°C since the released virus is inactivated within 3 d in a cell-free medium at this temperature. Under natural conditions, the virus is thus likely to be quickly inactivated in the aquatic environment when exposed to temperatures above 30°C.

In developing countries such as the Philippines, where electrical shut downs are not uncommon, optimum storage temperature of the virus must be determined. The results showed the limitations of virus storage in the absence of an ultralow temperature freezer. Storage of the virus at higher temperatures of –10 and 8°C should not exceed 30 and 10 d, respectively. These data also serve as a useful guide to temperatures needed during sampling and transport of the virus.

A birnavirus (Hedrick et al. 1986), several rhabdoviruses (Frerichs et al. 1989, Kasornchandra et al. 1991, Lilley & Frerichs 1994) and a reovirus (Roberts et al. 1994) have previously been isolated from EUS-affected fishes in Thailand, Myanmar and Sri Lanka. The birnavirus and the reovirus isolations were reported only

once, whereas rhabdoviruses have been isolated several times from EUS-affected fish. Hence, the association of rhabdoviruses with the etiology of EUS requires further study.

Morphologically, both the UDRV and the 91-97 virus have typical bullet-shapes with blunt bases and rounded opposite ends while SHRV is bacilliform with both ends rounded.

Differences in susceptibility of various cell lines to 91-97, UDRV and SHRV were noted. Atlantic salmon cells were refractory to infection with the present isolate while UDRV replicated to high titers in it (Frerichs et al. 1989). CHSE-214 cells were refractory to UDRV while low titers of the present isolate (10^3 TCID$_{50}$ ml^{-1}) were produced in CHSE-214 cells. Moreover, EPC cells were refractory to both the present isolate and UDRV, while SHRV replicated optimally in them (Frerichs et al. 1989, Kasornchandra et al. 1991).

Serological tests showed the UDRV antiserum cross-reacted slightly with the 91-97 virus but not with SHRV. This indicates that the 91-97 virus isolate is antigenically distinct from SHRV but somewhat related to UDRV. The very slight cross-reaction of the 91-97 virus antiserum with IHNV is considered insignificant since the IHN antiserum did not cross-react with the test virus. Other workers have also shown the serological unrelatedness of UDRV, SHRV and IHNV (Kasornchandra et al. 1992).

By and large, the foregoing morphological, growth, biochemical and cytopathological characteristics of the 91-97 virus and the antigenic relationships obtained from the present study indicate similarities and dissimilarities with the UDRV. Further studies, however, are required to determine the degree of the relationship.

Infection experiments failed to induce pathologic effects in healthy snakeheads. Frerichs et al. (1993) reported similar observations using snakehead fish rhabdovirus and the snakehead cell line retrovirus isolated from EUS fish in Thailand by IP injection and by bath. However, virus was recovered from fish visceral organs until Day 8 post-inoculation for IP injected virus and Day 5 for other test routes of the virus, indicating that the virus remained viable in the inoculated fish, having been constantly recovered predominantly from the gonad and kidney and occasionally from the liver, spleen and gill suggesting its predilection to these organs.

In view of the results obtained with the challenged fish, further studies are needed to determine the role, if any, of the virus in the pathogenesis of EUS. There is a possibility that the isolate used in the challenge studies was partially attenuated because it had been passaged quite often in tissue culture before being used for the challenges. We intend, therefore, to investigate freshly isolated strains of the virus for their connection with EUS as soon as they become available. While we do not think it likely that a rhabdovirus would account for the clinical signs associated with EUS, we consider it possible that a rhabdovirus may be the primary agent involved in predisposing fish to other organisms that may be the actual causes of EUS-type lesions.

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