

# Virus Isolation from Epizootic Ulcerative Syndrome-Diseased Fishes

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## Abstract

Epizootic Ulcerative Syndrome (EUS) is a seasonal and widely spread ulcerative condition of fresh and brackishwater fishes in Asia caused by a complex of etiological agents. Viral agents have been found to be associated with EUS, but their role in the complex etiology still has to be identified. Further virological examinations were, therefore, conducted in this study. Twenty viral isolations were achieved from three epizootics in Thailand. In the 1993-1994 epizootic, nine rhabdoviruses were successfully isolated from EUS snakehead (*Channa striata*) and three-spot gourami (*Trichogaster trichopterus*). Another nine rhabdoviruses were also isolated from 11 tissue extracts from affected snakeheads collected in the 1995-1996 outbreak. Two other viral isolates were obtained from affected giant gourami (*Osphronemus gouramy*) and climbing perch (*Anabas testudineus*) in the 1996-1997 outbreak. These viruses were isolated only from samples collected during the early period of the outbreaks. The tissue samples collected from moribund fish, processed and inoculated on to cell culture within a few days gave the best virus isolation results. Virus isolation from the samples collected after the early period of the EUS from Thailand and Pakistan gave no cytopathic effect on the host cells. In this study, BF-2 cells seemed to be the best cell line for viral isolation followed by SSN-1 and HCK lines, while no virus could be obtained using the EPC and BB lines. The results of this study suggest that the viruses, especially rhabdovirus, are readily isolated from diseased fishes during the early period of an outbreak, and that they may have a significant role in a complex of etiological agents of EUS.

## Introduction

EUS is a seasonal condition that usually occurs after heavy rains and during the cooler part of the year and causes ulcerative lesions on the body and/or head of the affected fish. The primary causative agent is still unknown and a complex of etiological agents is suggested to be involved. A pathogenic fungus, *Aphanomyces invaderis*, was isolated from the muscle tissue beneath the ulcers of EUS-affected striped snakehead fish (*Channa striata*) in 1992 (Willoughby et al., 1995). This fungus was later renamed *Aphanomyces invadans* by Lilley et al., (1997). The fungus causes disease in snakehead only through injection or

insertion of its spores or fungal hyphae into the body of the fish; a bath challenge of the fish with fungal spores causing no disease (Chinabut et. al., 1995).

Viruses have been found to be associated with EUS as can be seen in early reports of successful viral isolation in the 1980s. Prior to the present study, eight rhabdoviruses, four birnaviruses and one reovirus have been isolated (Table 1). Previous viral infection experiments indicated that viruses cause only minor skin damage with a low level of haemorrhage (Frerichs et. al., 1993). Although a virus is believed to be a primary causative agent, the percentage of successful viral isolation was very low (Roberts et. al., 1994; Frerichs, 1995). Further isolation studies from four EUS outbreaks in Thailand and one outbreak in Pakistan were carried out in order to determine the significance of viruses in EUS outbreaks.

## Materials and Methods

### *Tissue preparation and viral isolation*

Tissue preparation was similar to the general procedures stated elsewhere (Hetrick, 1989). Approximately 1 gm of a pooled tissue was taken from the muscle and/or internal organs (kidney, liver, spleen, intestine and pancreas). The sample was homogenised in a precooled pestle and mortar, diluted with 9 mL Hanks' balanced salt solution (HBSS) containing 2% foetal calf serum and spun at 800'g at 4°C for 15 min. The clear extract was diluted to 1:50 then filter-sterilised through 0.45 mm membrane filter units. Simultaneous cell culture and sample inoculation were carried out using BF-2, SSN-1 (Frerichs et. al., 1991), HCK (Kanchanakhan & Frerichs, 1995), BB and/or EPC cell lines. Tests were conducted in 24-well plates. Cells were incubated at 23-25°C and observed daily, and a first blind passage of culture fluids was performed on days 7-10. Transmittable cytopathic effect (CPE) for two passages was considered positive viral isolation.

### *Viral morphology*

Viruses were propagated in 25 cm<sup>2</sup> flask of fathead minnow (FHM) cell lines. The cell sheet was fixed using Karnovsky's fixative and transferred to a centrifuge tube then spun at 1100'g at 4°C for 10 min. The packed cells with viruses were processed for transmission electron microscopy examination according to Hayat (1989). Grids were observed using a Phillips 301 electron microscopy at 80 kV.

### *EUS outbreaks in Thailand and Pakistan*

#### EARLY PERIOD EUS

Fish samples were collected from five provinces in Thailand at about one to three weeks after the start of the outbreak. Fish showed multiple shallow

Table 1. Viruses isolated from EUS-affected fish (from Frerichs (1995) with some modifications).

Viral agent	Fish host and country	Date of collection or viral isolation	References
<b>Birnaviruses</b>			
Sand goby virus (SGV)	Cage-cultured marble goby ( <i>Oxyeleotris marmorata</i> ), Ayutthaya Province, Thailand	March 1984	Hedrick et al., 1986
Snakehead fish virus (SHV)	Many fish species from Thailand	January-March 1983	Saitanu et al., 1986
IPNV similar to serotype Sp	Pooled samples from two snakehead fish (one from Thailand and one from Myanmar) and one eye-spot barb ( <i>Hampala dispar</i> ) from Lao P.D.R.	December 1985-January 1986	Wattanavijarn et al., 1988
IPNV different to Vr-299, Sp and Ab serotype	Giant snakehead ( <i>C. micropeletes</i> ), Singapore		Subramanian et al., 1993
<b>Reovirus</b>			
Reovirus-like	Snakehead, Suphanburi, Thailand	January-February 1992	Roberts et al., 1994
<b>Rhabdoviruses</b>			
Snakehead rhabdovirus (SHRV)	Snakehead, Nakhonpathom, Thailand	December 1985-January 1986	Wattanavijarn et al., 1986; Kasornchandra, et al., 1991
Rhabdovirus T9204	Snakehead, Suphanburi, Thailand	January 1992	Roberts et al., 1994; Lilley & Frerichs, 1994
Ulcerative disease rhabdovirus (UDRV) strains BPV, O <sub>2</sub> , 19, 20E	Snakehead and swamp eel ( <i>Fluta alba</i> ) from Thailand and Myanmar	October-December 1985	Frerichs et al., 1986, 1989a
UDRV strain SL11	Snakehead from Sri Lanka	February 1988	Frerichs et al., 1989b
Rhabdovirus A4	Blackfish ( <i>Girella tricuspidata</i> ), Myall Lake, NSW, Australia	December 1985	Lilley & Frerichs, 1994

ulcers, pink to red in color and light-brown filamentous material on the surface.

#### MIDDLE PERIOD EUS

Fish samples were collected from two provinces in Thailand and Salkot District, Punjab, Pakistan, four to six weeks after the start of the outbreak.

#### LATE PERIOD EUS

Fish samples were collected from Prachinburi, Thailand six weeks after the start of the outbreak.

#### RECOVERY PERIOD EUS

Recovery period EUS samples were collected from Suphanburi and Bangkok, Thailand, after the disease had apparently disappeared.

Live specimens were collected and transported in an air-conditioned mini-truck to the Virology Unit at the AAHRI, where tissue extracts were immediately prepared for viral isolation. For a longer distance transport, 1 gm of tissue was taken from the specimen and transported to the laboratory in HBSS supplemented with 2% serum and 500 IU/mL penicillin, 500 mg/mL streptomycin and 10 mg/mL amphotericin-B in a cool box. For the Pakistani samples, a hand centrifuge was used instead of an electrical refrigerated centrifuge and the samples were spun by hand for 10-15 min. The samples have been diluted 1:50, filtered and held in sterile bottles in a cool box or refrigerator for seven days before the extracts were inoculated on to fish cell culture. The sample codes and details are given in Table 2.

## Results

### *Early period EUS*

Viral isolation were achieved from three epizootics in Thailand. During the early period EUS, sample codes AV9404, AV9405, AV9601, AV9701 and AV9702 gave positive CPE in BF-2; while sample codes AV9402 and AV9501 did not give any CPE (Table 2). All viruses were isolated from moribund fish except the sample code AV9405 for which one virus was isolated from dead fish. Sample codes AV9402 and AV9405 were collected from the same fish pond, however, the former was collected a week before the latter. Six fish of the former code and three fish of sample code AV9501 died during transportation and no virus could be isolated. BF-2 was found to be the best line for viral isolation followed by SSN-1 and HCK lines. No virus was isolated using BB and EPC lines. The isolated viruses, except AV9701 and AV9702, were identified as rhabdoviruses, as their morphology (Fig. 1), physico-chemical properties and structural proteins in gel-electrophoresis were similar. The viruses AV9701

Table 2. Details of viral isolation from diseased and normal fishes collected at different periods of the EUS outbreaks in Thailand and Pakistan.

EUS condition	Sample code (Year)	Location	Fish species and weight	D = diseased N = normal	Virus isolate obtained
Early period (a few weeks)	AV9402 (1994)	Suphanburi (site 1)	6 snakehead ( <i>Channa striata</i> )	D	None*
	AV9404 (1994)	Bangkok	6 snakehead	D	4 rhabdoviruses**
			3 three-spot gourami ( <i>Trichogaster trichopterus</i> )	D	1 rhabdovirus
	AV9405 (1994)	Suphanburi (site 1)	7 snakehead	D	4 rhabdoviruses***
	AV9501 (1995)	Nonthaburi	3 snakehead	D	none
	AV9601 (1996)	Pichit	6 snakehead	D	9 rhabdoviruses
	AV9701 (1997)	Lopburi	9 snakehead 3 climbing perch ( <i>Anabas testudinus</i> )	D D	1 virus from climbing perch
	AV9702 (1997)	Bangkok	2 giant gourami ( <i>Osphronemus goramy</i> )	D	1 virus
Mid period (4-6 weeks)	AV9408 (1994)	Suphanburi (site 2)	6 snakehead fish	D	none
	AV9705 (1997)	Nakornrajsima	3 climbing perch	D	none
			1 climbing perch	N	
			1 snakehead fish	D	
			2 marble goby ( <i>Oxyeleotris marmorata</i> )	D	
			1 pipe nose fish ( <i>Microphis boaja</i> )	N	
			4 spotted spiny eel	D	
	AV9707 (1997)	Punjab, Pakistan****	2 siver carp ( <i>Hypophthalmichthys molitrix</i> )	D	none
			3 grass carp ( <i>Ctenopharyngodon idella</i> )	D	
			3 rohu ( <i>Labeo rohita</i> )	D	
1 mrigal ( <i>Cirrhinus mrigala</i> )			D		
1 pool barb ( <i>Puntius sophore</i> )			D		
1 catla ( <i>Catla catla</i> )			D		
1 commom carp ( <i>Cyprinus carpio</i> )			D		
Late period (>6 weeks)	AV9412 (1994)	Prachinburi	2 snakehead	D	None
Recovery period	AV9420 (1994)	Suphanburi (site 1)	22 snakehead	N	none
	AV9421 (1994)	Bangkok	5 snakehead	N	none

\* Dead EUS fish gave no virus isolation.

\*\* Moribund fish gave five rhabdovirus isolates.

\*\*\* Five dead EUS fish gave one rhabdovirus, while four extracts from two moribund fish gave three rhabdoviruses.

\*\*\*\* Only *Puntius sophore* was histologically identified as EUS-affected.

and AV9702 possessed genomic RNA and had a tiny spherical particle with an envelope derived from host cell membrane (S. Kanchanakhan, unpublished data).

### *Middle, late and recovery EUS period*

No virus could be isolated from samples collected during middle (codes AV9408, AV9705, AV9707), late (AV9412) and recovery periods (AV9420, AV9421) of EUS in Thailand and Pakistan. All tissue extracts gave no CPE on indicator cell lines at initial inoculation, 1st blind and 2nd blind passages.

## Discussion

Viruses were successfully isolated only from the diseased samples collected during the early period EUS. Nineteen viruses were obtained from moribund specimens, while only one virus was isolated from dead fish. Many isolation attempts made during the middle, late and recovery period of EUS failed. BF-2 cell line was found to be the best line for viral isolation in this study.

As a result of the isolation studies, 18 isolates belonging to a single family, the Rhabdoviridae, were obtained, while two other isolates belonging to other families were also encountered. More characteristics and properties are needed in order to identify these two isolates from climbing perch (*Anabas*



Fig. 1. A micrograph of one virus isolate from EUS-affected snakehead fish collected from Bangkok. The virus is identified as Rhabdovirus, ~53 nm in diameter and ~177 nm in length.

*testudineus*) and giant gourami (*Osphronemus gouramy*). Rhabdoviruses seem to predominate in diseased snakehead, as some previous isolates were also obtained from this fish or closely related species of Channidae (Roberts et. al., 1994; Wattanavijarn et. al., 1986; Frerichs et. al., 1986 and 1989a and 1989b). The presence of viruses in the diseased fishes during the early period of the outbreaks indicates that virus may play a significant role in the complex etiology. In general, each virus has a specific host range; thus virus is unlikely to be the primary infectious agent for over 100 fish species that have been recorded as susceptible to EUS in Asia (Lilley et. al., 1992). It is therefore possible that viruses may be one part of the complex causative agents that break the outer protection of the skin of the fish.

Previously, viruses have been disregarded as possible primary causative agents of EUS, because of the low frequency of viral isolation from EUS-infected fishes collected from Southeast Asia, and the fact that no virus was isolated from a large number of diseased fish samples during the major EUS outbreak in Bangladesh (Roberts et. al., 1994; Frerichs, 1995). The low overall frequency of viral isolation in their studies might have been due to sampling after the early period of the outbreak, as no viruses were isolated from the samples collected after the early period of EUs in this study. These findings are in general agreement with those from isolation attempts for other rhabdoviral diseases of fish, such as VHSV, which has been shown to be isolated from diseased fish during an EUS but not from the surviving fish (Wolf, 1988). The amount of isolatable viruses in the diseased fish decreased after the start of the outbreak, and similar events were also demonstrated in experimental infections with IHNV (Bootland et. al., 1994), SVCV (Ahne, 1986) and IPNV (Frantsi & Savan, 1971). However, the onset and duration of EUS outbreaks may vary from year to year and from country to country and are likely to be directly related to the duration of cool seasons. The period of successful viral isolation may therefore also vary from one outbreak to another. Furthermore, fish had to be alive immediately before sampling to achieve the best viral isolation results. This finding agrees with the suggested general technique for specimen collection provided by Hetrick (1989).

The repeated success of viral isolation attempts made during the early period of the EUS suggests that virus may play an important role in the complex etiology. Recent information shows that rhabdoviruses can cause death in snakehead fry and skin damage to juveniles. Snakeheads that had previously been injected with rhabdovirus then given a bath challenge with *A. invadans* showed severe clinical signs of EUS in the spores (Kanchanakhan, 1996).

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## References

- Ahne, W. (1986). The influence of environmental temperature and infection route on the immune response of carp (*Cyprinus carpio*) to spring viremia of carp virus (SVCV). *Veterinary Immunology and Immunopathology*. 12:383-386.
- Bootland, L.M., Lorz, H.V., Rohovec, J.S. & Leong, J.C. (1994). Experimental infection of brook trout with infectious haematopoietic necrosis virus type 1 and 2. *J. Aquat. Animal Health*. 6:144-148.
- Chinabut, S., Roberts, R.J., Willoughby, G.R. & Pearson, M.D. (1995). Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. *J. Fish Dis.* 18:41-47.
- Frantsi, C. & Savan, M. (1971). Infectious pancreatic necrosis virus: temperature and age factors in mortality. *J. Wildl. Dis.* 7:249-255.
- Frerichs, G.N. (1995). Viruses associated with the epizootic ulcerative syndrome (EUS) of fish in south-east Asia. *Vet. Res.* 26: 449-454.
- Frerichs, G.N., Hill, B.J. & Way, K. (1989a). Ulcerative disease rhabdovirus: cell-line susceptibility and serological comparison with other fish rhabdoviruses. *J. Fish Dis.* 12:51-56.
- Frerichs, G.N., Millar, S.D. & Alexander, M. (1989b). Rhabdovirus infection of ulcerated fish in South-East Asia. In *Viruses of Lower Vertebrates* (Ahne, W. & Kurstak, E., eds.) pp. 396-410. Springer-Verlag, Berlin.
- Frerichs, G.N., Millar, S.D. & Chinabut, S. (1993). Clinical response of snakeheads (*Ophicephalus striatus*) to experimental infection with snakehead fish rhabdovirus and snakehead fish cell line retrovirus. *Aquaculture*. 116: 297-301.
- Frerichs, G.N., Millar, S.D. & Roberts, R.J. (1986). Ulcerative rhabdovirus in fish in South-East Asia. *Nature Lond.* 322: 216.
- Frerichs, G.N., Morgan, D., Hart, D., Skerrow, C., Roberts, R.J. & Onions, D.E. (1991). Spontaneously productive C-type retrovirus infection of fish cell lines. *J. gen. Virol.* 72: 2537-2539.
- Hayat, M.A. (1989). *Principles and Techniques of Electron Microscopy: Biological Applications*, 3<sup>rd</sup> edn. MacMillan Press, London.
- Hedrick, R.P., Eaton, W.D., Fryer, J.L., Groberg, W.G. Jr. & Boonyaratpalin, S. (1986). Characteristics of a birnavirus isolated from cultured sand goby, *Oxyeleotris marmoratus*. *Dis. aquat. Org.* 1:219-225.
- Hetrick, F.M. (1989). Fish viruses. In *Methods for the Microbiological Examination of Fish and Shellfish* (Austin, B. & Austin, D.A., eds.) pp. 216-239. Ellis Horwood Ltd., Chichester, UK.
- Kanchanakhan, S. (1996). *Field and Laboratory Studies on Rhabdoviruses Associated with Epizootic Ulcerative Syndrome (EUS) of Fishes*. Ph.D. Dissertation, University of Stirling, Scotland.
- Kanchanakhan, S. & Frerichs, G.N. (1995). Two established cell lines derived from head kidney and tail of hybrid catfish (*Clarias gariepinus* X *C. macrocephalus*). In: *Diseases in Asian Aquaculture II* (Shariff, M., Arthur, J.R. & Subasinghe, R.P., eds.) pp. 369-383. Fish Health Section, Asian Fisheries Society, Manila.
- Kasornchandra, J., Lannan, C.N., Rohovec, J.R. & Fryer, J.L. (1991). Characterization of a rhabdovirus isolated from the snakehead fish (*Ophicephalus striatus*). In *Proceedings of the Second International Symposium on Viruses of Lower Vertebrates* (Fryer, J.L., ed.) pp. 175-182. Oregon State University, Corvallis, Oregon.
- Lilley, J.H. & Frerichs, G.N. (1994). Comparison of rhabdoviruses associated with epizootic ulcerative syndrome (EUS) with respect to their structural proteins, cytopathology and serology. *J. Fish Dis.* 17:513-522.
- Lilley, J.H., Hart, D., Richards, R.H., Roberts, R.J., Cerenius, L. & Soderhall, K. (1997). Pan-Asian spread of single fungal clone results in large scale fish kills. *Veterinary Record*. 140: 653-654.
- Lilley, J.H., Phillips, M.J. & Tonguthai, K. (1992). *A Review of Epizootic Ulcerative Syndrome (EUS) in Asia*. Aquatic Animal Health Research Institute and Network of Aquaculture Centres in Asia, Bangkok.
- Roberts, R.J., Frerichs, G.N., Tonguthai, K. & Chinabut, S. (1994). Epizootic ulcerative syndrome of farmed and wild fishes. In *Recent Advances in Aquaculture, Vol. 5* (Muir, J.F. & Roberts, R.J., eds.) pp. 207-239. Blackwell Science Ltd., Oxford, UK.

- Roberts, R.J., Willoughby, L.G. & Chinabut, S. (1993). Mycotic aspects of epizootic ulcerative syndrome (EUS) of Asian fishes. *J. Fish Dis.* 16:169-183.
- Saitanu, K., Wongsawang, S., Sunyascotcharee, B. & Sahaphong, S. (1986). Snakehead fish virus isolation and pathogenicity studies. In *The First Asian Fisheries Forum* (Maclean, J.L., Dizon, L.B. & Hosillos, L.V., eds.) pp. 327-330. Asian Fisheries Society, Manila, Philippines.
- Subramanian, S., Chew-lim, M., Chong, S.Y., Howe, J., Ngoh, G.H. & Chan, Y.C. (1993). Molecular and electron microscopic studies of infectious pancreatic necrosis virus from snakehead. *IXth International Congress of Virology*. Glasgow, UK (Abstract), p. 368.
- Wattanavijarn, W., Tangtrongpiros, J. & Wattanodorn, S. (1986). Viruses of ulcerative diseased fish in Thailand, Burma and LAO P.D.R. In *First International Conference on the Impact of Viral Diseases on the Development of Asian Countries*. WHO. (Abstract) p. 121.
- Wattanavijarn, W., Torchy, C., Tangtrongpiros, J. & de Kinkelin, P. (1988). Isolation of a birnavirus belonging to Sp serotype, from Southeast Asia fishes. *Bull. Eur. Ass. Fish Pathol.* 8(5):106-108.
- Willoughby, G.R., Roberts, R.J. & Chinabut, S. (1995). *Aphanomyces invaderis* sp. nov., the fungal pathogen of freshwater tropical fish affected by epizootic ulcerative syndrome. *J. Fish Dis.* 18:273-275.
- Wolf, K. (1988). *Fish Viruses and Fish Viral Diseases*. Cornell University Press, Ithaca, New York.