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STUDIES ON SEVERAL VIRUS ISOLATES, BACTERIA AND A FUNGUS ASSOCIATED WITH EPIZOOTIC ULCERATIVE SYNDROME (EUS) OF SEVERAL FISHES IN THE PHILIPPINES

by

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ABSTRACT

Studies were conducted to determine the causative agent/s of Epizootic Ulcerative Syndrome (EUS) in snakeheads (*Ophicephalus striatus*) and catfish (*Clarias batrachus*). Results showed that EUS could be transmitted to naive snakeheads or catfish by cohabitation with EUS-diseased and apparently healthy snakeheads.

Severely EUS-lesioned snakeheads showed a mean bacterial load of 1.14×10^7 colony forming units (cfu) per gram of lesioned tissue, predominantly *Aeromonas hydrophila*. This bacterium induced dermomuscular lesions upon intramuscular (IM) injection of at least 10^5 cfu per catfish or snakehead.

Viruses were isolated from 35 % of 71 EUS-affected fishes, predominantly from moderately lesioned fish. A virus titer of 3.02×10^6 $TCID_{50}$ per ml was attained in snakehead spleen (SHS) cells at 25 °C with optimum replication at 15 °C. The virus was inactivated at 56 °C for 30 min, resistant to IUDR and sensitive to chloroform and low pH. Freeze-thaw and storage at -10 and 8 °C for 10 days did not significantly affect titer. Humoral viral neutralizing antibodies of snakeheads showed a decreasing trend in mean viral titer with severity of the disease.

The virus induced dermal lesions (+ to +++) 3 to 10 days following IM injection of at least 10^3 $TCID_{50}$ per fish to naive snakehead juveniles at 20 - 22.5 °C but not at 28 - 32 °C. Naive snakehead fry and fingerlings exposed to the virus by bath manifested significant mortalities ($p<0.01$) on days 4 and 5 with no apparent lesions. When the 95-96 virus isolate, *A. hydrophila* and *Aphanomyces* sp. were inoculated individually into the muscles of naive snakehead juveniles, +++, ++++ and + to ++ lesions were induced,

respectively. Addition of *A. hydrophila* to a previously virus-injected fish, however, exacerbated the virus-induced lesion to ++++. Treatments with virus plus bacteria, virus plus fungus and all three pathogens together resulted in histopathologies consistent with those induced by virus/bacteria.

Virus isolated and primarily propagated in SHS cells (91-97) was non-pathogenic while that isolated and primarily propagated in channel catfish ovary (CCO) cells (95-96) was pathogenic to naive snakeheads. This difference appears related to the generation of defective interfering (DI) viral particles by the former. Both isolates showed typical rhabdoviral morphology; dimensions of 65 x 175 nm; similarity of SDS-Page protein bands except for an additional band (approximate molecular weight = 29 kD) in 95-96; and antigen sharing, hence, are nearly related rhabdoviruses. The name EUS rhabdovirus (ERV) is proposed.

Taken together the data indicate that the rhabdovirus described here is likely the primary pathogen of EUS with *A. hydrophila*/*Aphanomyces* sp. as secondary infectious agents.

DEDICATED

To

Mama, Pol, Paolo, Emil, Patricia and Stephen

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CHAPTER 1

GENERAL INTRODUCTION

The initial occurrence of ulcerative epizootics affecting wild and cultured snakeheads (*Ophicephalus striatus*), catfish (*Clarias* sp.), climbing perch (*Anabas* spp.), spiny eel (*Mastamcembelus armatus*) and gourami (*Trichogaster* spp.) were observed in Malaysia in 1979 (Shariff and Law 1980). The following year, ulcerative epizootics of similar fish species including barbs (*Puntius* spp.) were found in Indonesia (Dana 1987). About the same time, serious outbreaks were reported in Thailand; these caused severe mortalities among snakeheads, serpent fish (*Channa micropeltes*), sand goby (*Oxyeleotris marmoratus*), barbs, three-spot gourami (*T. trichopterus*), striped croaking gourami (*Trichopsis vittatus*), siamese fighting fish (*Betta splendens*), wrestling half-beak (*Dermogenus pustillus*), swamp eels (*Fluta alba*) and several wild fish species (Saitanu *et al.* 1986; Tonguthai 1985). The subsequent spread of this epizootic in Myanmar (Boonyaratpalin 1985), Kampuchea and Lao PDR (Tonguthai 1985) in 1984, indicated that this disease had become widespread (Table 1.1). Subsequently, other countries reported the occurrence of epizootic ulcerative syndrome (EUS): Philippines in

Table 1.1. Location and first reported outbreaks of the epizootic ulcerative syndrome (EUS) in Asian countries.

First Outbreak	Country	Reference
January 1973	Vietnam	Tuan 1994
1977	Singapore	Roberts 1986
1979	Malaysia	Shariff and Law 1980
Late 1980	Indonesia	Dana 1987
Late 1980	Thailand	Tonguthai 1985
1984	Kampuchea	Tonguthai 1985
1984	Myanmar	Boonyaratpalin 1985
Early 1984	Lao PDR	Tonguthai 1985
December 1985	Philippines	Mines and Baluyot 1986
1987	Sri Lanka	Boonyaratpalin 1988
February 1988	Bangladesh	Barua 1989
May 1988	India	Jhingran 1988
1989	Bhutan	Phillips 1989
February 1989	Nepal	Shresta 1990

1985 (Mines and Baluyot 1986), Sri Lanka in 1987 (Boonyaratpalin 1988; Costa and Wijeyaratne 1989), Bangladesh in 1988 (Barua 1989), India in 1988 (Jhingran 1988), Nepal in 1989 (Shresta 1990 and 1994) and Bhutan in 1989 (Phillips 1989). Later reports indicated that this disease was also observed as early as 1973 and 1977 among catfish in Vietnam (Tuan 1994) and Singapore (Roberts 1986), respectively.

EUS-like lesioned fishes were also reported in Australia in 1972 (Rodgers and Burke 1977) affecting mullet (*Liza* spp.; *Mugil* sp.), whiting (*Silago* sp.), bream (*Aeathopagurs australia*) and snob nose garfish (*Arramphus sclerolepis*). The disease was then called Red Spot Disease (RSD). Similarly, in Papua New Guinea archerfish (*Toxotes chatareus*), nursery fish (*Kurtus gulliveri*), gudgeons (*Oxyeleotris* sp.; *Bunaka* spp.), gobies (*Glossogobius guirus*), freshwater anchovy (*Scutengraulis seratchlevi*) and scat (*Scatophagus argus*) were reported severely affected in 1975 (Haines 1983). In Southern China, large-scale outbreaks of EUS were not reported. However, there were reported occurrences of RSD-like lesions among crucian carps (*Carassius auratus*) and Indian major carps (*Labeo rohita*) in 1982.

Fishes affected with EUS were found in all types of freshwater environments, including lakes, rivers, streams, culture ponds, rice paddies, irrigation canals and reservoirs. In the Philippines, ulcerative lesions were initially observed among snakeheads, catfish and other wild species of freshwater fishes in Laguna de Bay (Mines and Baluyot 1986). In 1986, this disease was named Epizootic Ulcerative Syndrome (EUS) (Roberts *et al.* 1986).

In the Philippines, the occurrence of EUS was observed first in the largest freshwater lake in the country, Laguna de Bay, in late 1985 (Mines and Baluyot 1986).

Subsequently, EUS was recorded in fish in rivers and freshwater ponds in other provinces of Luzon, in Leyte, in 1992, and very recently in Lake Lanao, in 1997 (Fig. 1.1). All outbreaks of EUS that were reported occurred from November to February each year. These times coincided with the coldest months of the year.

Lesions of both wild and cultured fishes due to EUS are characterized by severe, ulcerative, dermal necrosis with extensive erosion/sloughing of the underlying musculature (Fig. 1.2). The necrotic muscular tissue emits a foul odor. Fish manifest frank ulcers consisting of eroded dermal layer, exposing the underlying musculature, which may be hemorrhagic. Fish with less severe lesions exhibit scale loss with erosion of the skin surface with or without hemorrhagic signs (Lio-Po *et al.* 1992).

EUS affects many species of freshwater fishes, both wild and cultured. In the Philippines, species that commonly show this syndrome are snakeheads (*O. striatus*), catfish (*C. batrachus*), gourami (*T. trichopterus*, *T. pectoralis*), *goby (G. guirus*), silvery theraponid (*Therapon plumbius*) and climbing perch (*A. testudineus*). In other countries, other freshwater fishes that are severely affected include barbs (*Puntius* spp.) and serpent fish (*Channa* spp.). An estimated 48 fish species have been affected with EUS, mostly bottom or middle zone feeders. The most seriously affected fish genera in the 1989-90 EUS outbreak were barbs, snakeheads, catfish and spiny eels (Phillips and Keddie 1990). Many of the EUS-diseased wild and cultured finfishes die of the disease. Milkfish (*Chanos chanos*), tilapia (*Oreochromis* sp.) and bighead carp (*Aristichthys nobilis*) appear to be resistant to EUS (Llobrera and Gacutan 1987).

Severely EUS-affected fish that survive become disfigured and have no market value as foodfish. In addition, consumers have become wary of buying uninfected fishes

Fig. 1.1. Map of the Philippines indicating first reported epizootic ulcerative syndrome (EUS) outbreaks in descending order of year of occurrence (1) 1985-86 in Laguna de Bay, Laguna; (2) 1986-87 in Pangasinan; (3) 1988-89 in Bulacan (3a), Cagayan (3b), Catanduanes (3c), Isabela (3d), Ilocos Norte (3e), Naujan Lake, Mindoro Oriental (3f), Nueva Ecija (3g), Pampanga (3h), Quezon (3i), Tarlac (3j), Zambales (3k); (4) 1992-93 in Leyte; (5) 1997-98 in Lake Lanao, Lanao del Sur.

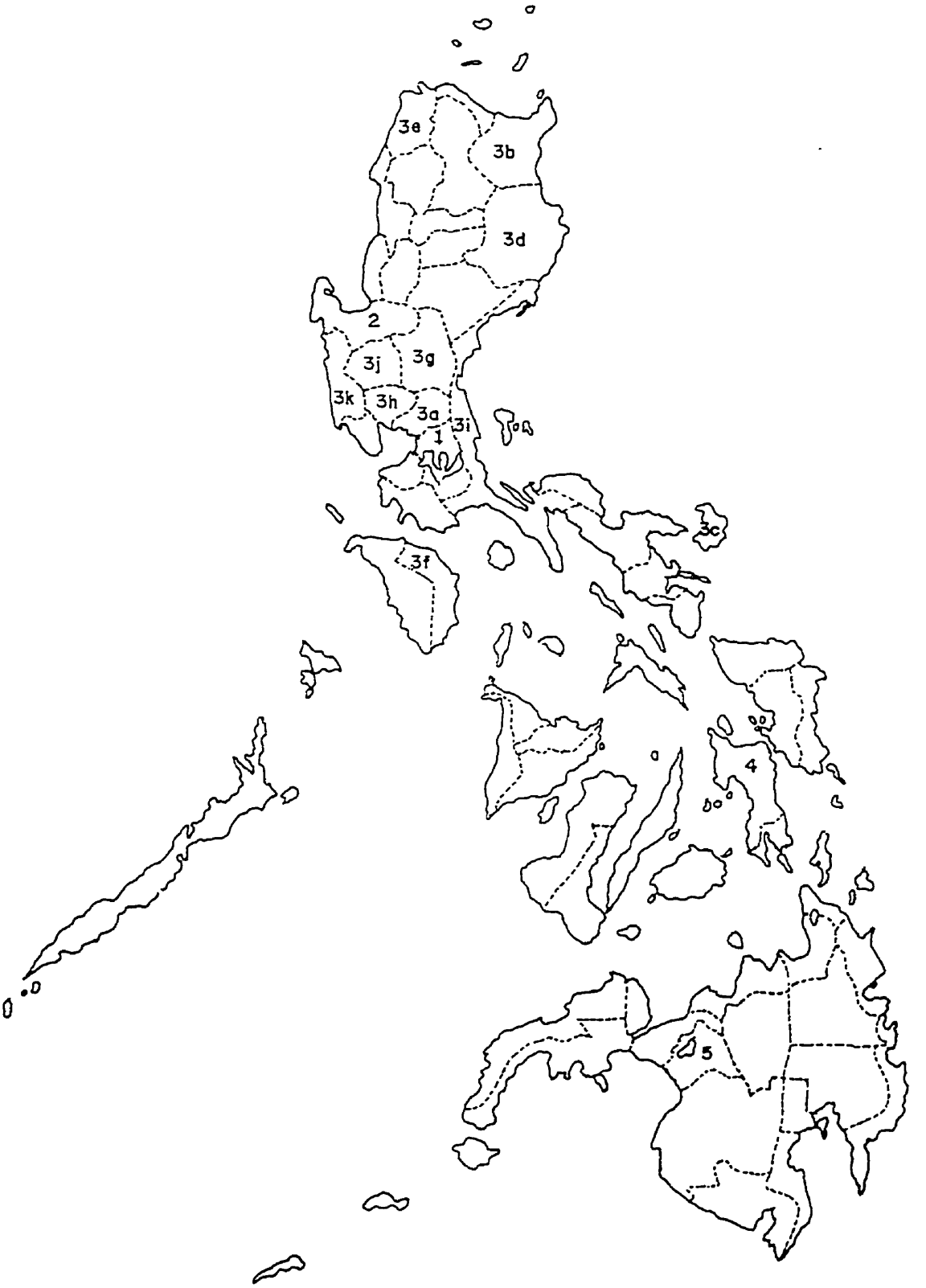
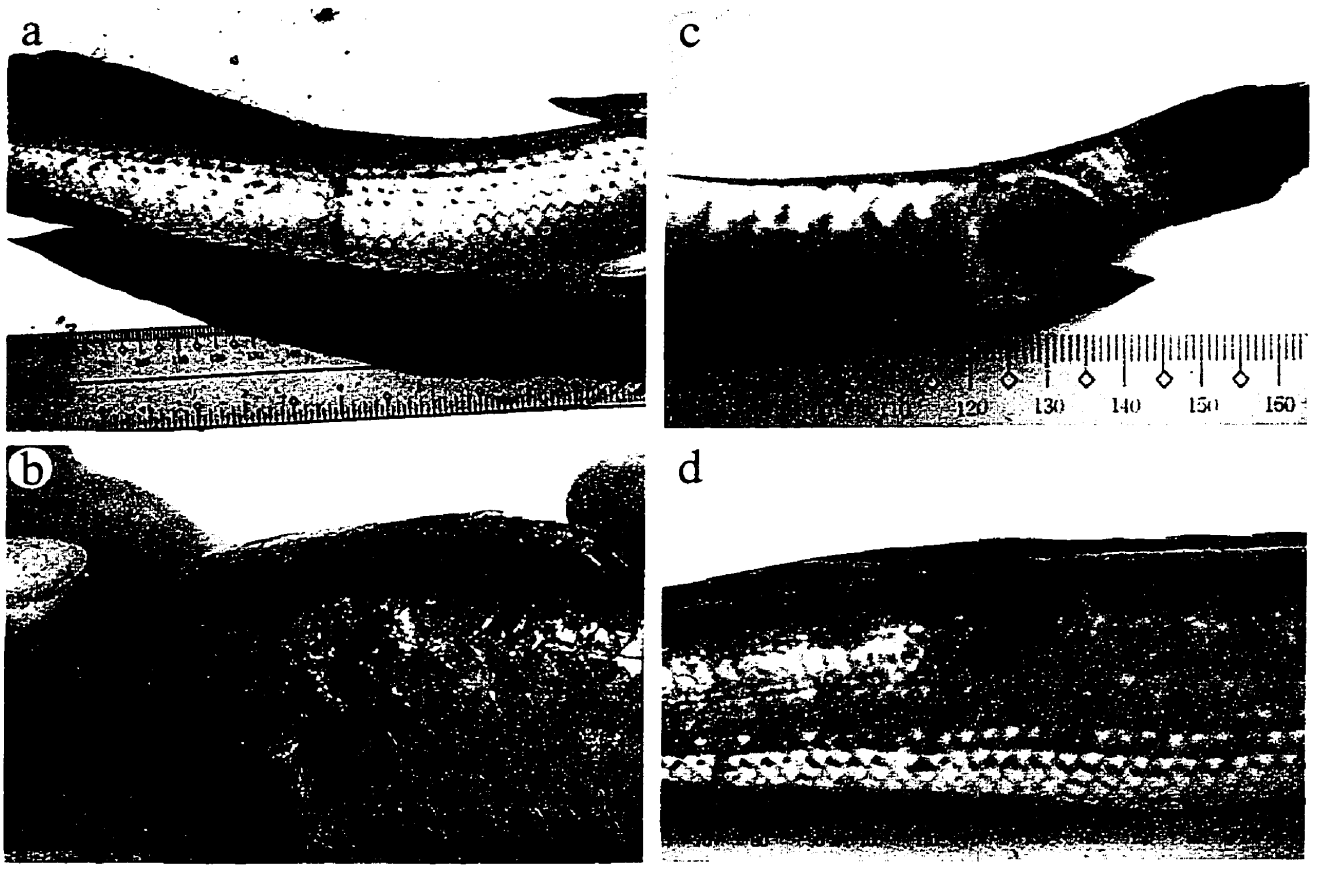

3e
3b
3d
2
3j
3g
3k
3h
3a
3i
1
3c
3f
4
5

Fig. 1.2. Epizootic ulcerative syndrome (EUS)-affected snakeheads (*Ophicephalus striatus*) with grade I (a), III (b), IV (c) and healed lesions (d).


a
c
b
d
120
130
140
150

whether or not they are known to be susceptible to EUS. As a result, their prices dropped by 30% in Laguna de Bay in 1985-86 (Llobrera 1987). Income losses to Filipino fish farmers were reported as much as 40 - 50 % in Pangasinan, and 40 - 60 % in Cagayan, during the 1989-90 and 1996-97 EUS outbreaks in the Philippines, respectively, (Bondad-Reantazo *et al.* 1994; Buencamino, Pangasinan, Philippines, pers. comm.). In Thailand, direct losses attributed to EUS were estimated at approximately US$ 8.7 million in the 1982-83 outbreak (Tonguthai 1985), exceeding US$ 100 million over the past decade (Chinabut 1994). In Bangladesh, 47 % of 257 fish ponds investigated were affected by EUS (Ahmed and Rab 1995). Prices of freshwater fish in that country decreased by 75% in 1988 and 1989 (Lilley *et al.* 1992) with estimated losses of US$ 3.38 and 2.24 million, respectively (Barua 1994). In India, 31 - 40 % losses of cultured fish were attributed to EUS. Thus, the economic impact of the disease in the Southeast Asian region is serious.

In Laguna de Bay, Philippines, the EUS morbidity rate among snakeheads was estimated to be 59 % in January 1986 (Mines and Baluyot 1986). In the 1988-1989 outbreak, infection rates of snakeheads, catfish and goby were observed as 76.4, 12.5 and 80 %, respectively (Palisoc 1990). In the 1995-96 annual outbreak of EUS among the fish catch in Laguna de Bay and a freshwater pond in Luzon, the prevalence was estimated to be 50 and 38 %, respectively. EUS outbreaks are continuing unabated (Filipino fishermen, pers. comm.).

In general, EUS outbreaks show a seasonal pattern (Phillips and Keddie 1990). Outbreaks are more common in the months of September to March, which correlate with the time when lowest water temperatures occur.

The spread of EUS outbreaks among populations of fish within affected countries and between affected countries strongly suggests the involvement of a microbial etiologic agent. Viral, bacterial, fungal, parasitic protozoans, helminths and crustaceans and environmental conditions associated with the syndrome have been investigated (Roberts *et al.* 1986). Pesticides and parasitic protozoans, helminths and crustaceans were ruled out as causes (Roberts *et al.* 1986; Phillips 1994). Consequently, the range of possible etiologic agents included viruses, bacteria and fungi (Roberts *et al.* 1986).

Viruses associated with EUS-affected fishes in Thailand, Myanmar and Sri Lanka included a reovirus or a picornavirus (Wattanavijarn *et al.* 1983; 1984), an IPN-like virus (Saitanu *et al.* 1986), a birnavirus (Hedrick *et al.* 1986), several rhabdoviruses (Frerichs *et al.* 1986; Frerichs 1989; Kasornchandra *et al.* 1991; Lilley and Frerichs 1994) and a reovirus-like agent (Roberts *et al.* 1994). Among these viruses, only the rhabdovirus was consistently isolated from EUS fish. However, upon experimental infection, Frerichs *et al.* (1993) and Millar (1994) could not demonstrate that these were pathogenic to healthy snakeheads.

Several bacteria were reported associated with EUS-affected fish e.g. *Aeromonas hydrophila*, *A. sobria*, *A. caviae*, and a *Pseudomonas* sp. (Boonyaratpalin 1989; Karunasagar et. al. 1994; Llobrera and Gacutan 1987; Pal and Pradhan 1990; Roberts *et al.* 1986; Torres *et al.* 1990). Among these, *A. hydrophila* appeared to be the most consistently isolated. *A. hydrophila* was also associated the "red spot" disease in sea mullet, *Mugil cephalus* (Burke and Rodgers 1981). It is also a common pathogen of channel catfish, *Ictalurus punctatus* (Figueiredo and Plumb 1977; Ventura and Grizzle 1988). However, its role as a pathogen has always been considered secondary.

Fungal hyphae were observed in tissue sections of EUS-affected fish (Lilley *et al.* 1992; Roberts *et al.* 1994; Cruz-Lacierda 1995). Subsequently, *Achlya* sp. and *Aphanomyces* sp. were isolated and reported as pathogenic (Lilley and Roberts 1997).

Cohabitation experiments with EUS-affected and healthy snakeheads conducted in natural waters during the EUS epizootic season in Sri Lanka and the Philippines, confirmed the transmissibility of EUS (Balasuriya *et al.* 1990; Cruz-Lacierda and Shariff 1995). Histopathological analyses of experimentally infected snakeheads showed tissue lesions similar to those in the naturally EUS-affected snakeheads.

If this disease is to be controlled, the causative agent must first be identified. To date, no microbe has been conclusively shown to be the primary agent. This research was therefore done to investigate the etiological role of several bacteria, viruses and fungi in EUS in certain finfish cultured in the Philippines.

CHAPTER 2

AEROMONAS HYDROPHILA IN THE EPIZOOTIC ULCERATIVE SYNDROME (EUS) OF CATFISH (*CLARIAS BATRACHUS*) AND SNAKEHEADS (*OPHICEPHALUS STRIATUS*): DETECTION DURING NATURAL INFECTIONS AND EXPERIMENTAL INDUCTIONS OF DERMO-MUSCULAR NECROTIC LESIONS

INTRODUCTION

In the Philippines, fish species affected by EUS are snakeheads (*O. striatus*), catfish (*C. batrachus*), guorami (*T. trichopterus*, *T. pectoralis*), goby (*G. guirus*) and climbing perch (*A. testudineus*). To date, the epizootiology of the disease has not been established. A virus was reported to be associated with lesions, and is perhaps the causative agent of EUS (Frerichs *et al.* 1986). Boonyaratpalin (1987) and Llobrera and Gacutan (1987) noted that *A. hydrophila* bacteria were often associated with EUS lesions. However, no conclusive experimental evidence of lesion induction in snakehead or catfish has been shown with a virus or bacterium. Therefore, experiments were done to define the role that bacteria play in the development of EUS.

MATERIALS AND METHODS

Sampling Sites

EUS-affected *C. batrachus* (75 - 135 g) and *O. striatus* (180 - 800 g) were collected from Laguna de Bay and freshwater ponds in Pangasinan and Pampanga (Table 2.1). Fish lesions were noted and classified at the time of sampling (Table 2.2). Healthy fish samples were taken from Iloilo, a non-enzootic area.

Bacterial Load Determination

Fish samples were externally disinfected with 70 % ethanol and aseptically dissected. Sections of fish kidneys and muscle lesions, lake/pond sediments, golden snails (*Ampullarius* sp.) and rearing waters were removed, weighed, homogenized in homogenizing tubes, serially diluted with sterile 0.85% physiologic saline (NSS) and inoculated onto trypticase soy agar (TSA) (BBL). Each 0.1 ml inoculum was thoroughly spread onto the medium using a sterile, bent glass rod. Snail samples were externally washed with sterile NSS and the snail tissue pulled out using sterile forceps. The tissues were then similarly processed as with fish tissues. Five samples of each were processed and inoculated in two replicates per dilution. After 24 - 48 h incubation at room temperature, total colony forming units (cfu) per g or ml sample were determined.

Bacterial Isolation and Identification

Clock streak inoculations of the above-mentioned samples were also done on

Table 2.1. Characteristics of several sampling sites in the Philippines that contain fish affected by epizootic ulcerative syndrome (EUS).

Location	Characteristic	Fish Species	
		EUS-Affected	Non-EUS-Affected
Laguna de Bay	Lake	*Anabas testudineus* *Clarias batrachus* *Glossogobius guirus* *Ophicephalus striatus* *Trichogaster pectoralis* *Trichogaster trichopterus*	*Cyprinus carpio* *Chanos chanos* *Oreochromis niloticus*
Pampanga	River-fed pond	*Ophicephalus striatus*	*Cyprinus carpio*
Pangasinan	Rain/Flood-fed pond	*Ophicephalus striatus*	

Table 2.2. Classification by lesion grade of fishes affected with the epizootic ulcerative syndrome (EUS) including apparently healthy fish.

Lesion Grade	Characteristic of Lesion
Normal	Fish from non-endemic area with no external signs of disease or lesions
Apparently Healthy	Fish from endemic area with no external signs of disease/lesions
Grade I	Scale loss with slight erosion of the skin surface with or without hemorrhagic spots
Grade II	Eroded dermal layer which may be hemorrhagic
Grade III	Frank ulcers consisting of eroded dermal layer, exposing the underlying musculature
Grade IV	Ulcers consisting of extensively eroded/sloughing underlying musculature at times emitting a foul odor

Rimler-Shotts medium. After 2 d incubation, selected and isolated bacterial colonies were scraped from Rimler-Shotts medium and from TSA, purified by restreaking on nutrient agar (NA) (Difco) and subsequently identified. Using results obtained from Gram staining and various biochemical and growth tests, the method of Collins and Lyne (1984) for identification of bacteria was used for preliminary classification of the isolates. Subsequently, the presumptive scheme of McCarthy (1975) for *A. hydrophila* identification and the identification guide of Popoff for *Aeromonas* (1984) were followed.

Infection Experiments

Healthy snakeheads (3 - 5 g and 15 - 25 g) and catfish (15 - 25 g) from an EUS-free area were collected and acclimated at the SEAFDEC Fish Health Laboratory, Iloilo. Pure cultures from selected *A. hydrophila* representing isolates from healthy and lesioned fishes, water and snails that were categorized into phenotypic groups by the preceding method, were suspended and serially diluted in NSS to the desired concentration before intramuscular injection into the epaxial musculature (0.05 - 0.1 ml to a depth of 0.25 - 0.5 cm) per test fish. Viable counts of each bacterial inoculum were determined by the spread plate method using serially diluted samples, inoculated onto TSA and incubated for 48 h at room temperature. Injection doses ranged from 10^4 to 10^{10} cfu per fish. Control fish were similarly injected but with NSS alone. Fish were stocked individually and maintained at 21 - 25 °C in 10-liter aquaria filled with UV-sterilized water. Two to five replicate fish were used for each treatment.

A similar infection experiment was also conducted using 10^5 and 10^7 inocula of different *A. hydrophila* isolates but with two sets of two replicates of inoculated fish reared at two temperatures: 22 - 25 °C and 30.5 - 31 °C.

Test fish were observed daily for 4 d for development of lesions and mortalities. Experimentally induced lesions were graded as in Table 2.3.

Statistical Tests

Total bacterial counts among muscle lesions of normal, apparently healthy and EUS-affected fish were tested for statistical significance following analyses of variance using the Duncan's multiple range test.

RESULTS

The viable bacterial number in the fish kidney samples varied from 7.70×10^1 to 1.87×10^4 cfu per g (Table 2.4). The number of bacteria in healthy fish was not statistically different from that in fish with EUS lesions. However, the mean bacterial load of 1.14×10^7 cfu per g in severely affected snakehead muscle/lesion differed significantly ($p<0.05$) from those of healthy and less lesioned fish. Among catfish, total cfu per g muscle/lesion differed significantly in healthy, apparently healthy and slightly lesioned fish.

The bacterial cfu in waters, sediments and golden snails varied from 3.25×10^3 - 3.15×10^4, 3.30×10^5 - 2.10×10^6 and 1.80×10^6 - 8.48×10^7, respectively. The bacterial content of golden snails was greater than that of water and underlying sediment.

Table 2.3. Criteria for assessing lesion grade in catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus*) when infected by *Aeromonas hydrophila*, epizootic ulcerative syndrome (EUS) rhabdovirus and/or *Aphanomyces* sp. under laboratory conditions.

Lesion Grade	Chararacteristic/s of Lesion
-	Normal, intact skin
-/+	Intact but melanized skin at injection site
+	Blanching/slight swelling at injection site
++	Blanching; furuncle-like lesion with dermal erosion with or without hemorrhagic periphery
+++	Extensive and blanched lesion with furuncle-like necrotic core
++++	Ulcerated lesion with underlying necrotic musculature

Table 2.4. Total bacterial counts (cfu) from the kidney and muscle/lesions of healthy, apparently healthy and epizootic ulcerative syndrome (EUS)-affected catfish *(Clarias batrachus)* and snakeheads (*Ophicephalus striatus*) sampled from Iloilo (non-EUS area) and from various EUS enzootic sites of Laguna de Bay, Pampanga and Pangasinan. The materials were plated on trypticase soy agar and then incubated at 25 °C for 2 d prior to counting.

Fish Species/ Condition	Source	cfu per gram tissue Kidney	Muscle/Lesions
Clarias batrachus			
Healthy	Iloilo	7.70×10	3.00×10^2 a*
Apparently Healthy	Laguna de Bay	5.95×10^3	4.30×10^4 b
Slightly Lesioned	Laguna de Bay	6.00×10^2	2.00×10^5 c
Ophicephalus striatus			
Healthy	Iloilo	2.77×10^3	5.48×10^2 a
Apparently Healthy	Laguna de Bay	1.19×10^4	1.66×10^3
	Pangasinan	6.00×10^2	0.00×10^3
	Pampanga	3.00×10^3	2.50×10^3
	(Mean)	7.34×10^3	1.22×10^3 a
Slightly Lesioned	Laguna de Bay	4.90×10^3	4.05×10^4
	Pangasinan	2.20×10^3	1.83×10^5
	Pampanga	-	-
Moderately Lesioned	Laguna de Bay	1.45×10^3	4.80×10^5
	Pangasinan	1.24×10^3	5.69×10^5
	Pampanga	-	-
	(Mean)	1.32×10^3	5.31×10^5 a
Severely Lesioned	Laguna de Bay	-	-
	Pangasinan	-	-
	Pampanga	1.87×10^4	1.14×10^7
	(Mean)	1.87×10^4	1.14×10^7 b

cfu colony forming units per gram tissue

\- no specimens

• counts with similar letter superscript are not statistically significant from each other after the Duncan's multiple range test

A total of 95 bacterial isolates were recovered from fish samples, culture water, lake/pond bottom sediment and golden snails; approximately 89 % of which were *A. hydrophila,* 6 % were Enterobacteriaceae and 5 % were *Pseudomonas* spp. *A. hydrophila* was invariably isolated from EUS-infected fish, waters, sediments and golden snails from Laguna de Bay, Pangasinan and Pampanga (Table 2.5). The prevalence of this bacterium in healthy fish from the non-enzootic area (Iloilo) ranged from 0 to 20 %. Prevalence of up to 100 % occurred in apparently healthy and EUS-affected fishes from the enzootic areas. In general, 90 % of snakeheads and 33 % of catfish specimens were positive for *A. hydrophila.*

Detailed biochemical and growth tests indicated that *A. hydrophila* isolates consisted of six phenotypic groups i.e. A, B, C, D, E and F (Table 2.6). Variations were noted in the utilization of serine, production of gas from glycerol and gas production in the triple sugar iron agar, reaction on Simmon's citrate, the methyl red, Voges-Proskauer and, lysine decarboxylase tests, and in the sensitivity to 0/129 and novobiocin.

Results of the pathogenicity tests are summarized in Tables 2.7 and 2.8. In *O. striatus*, dermal/muscular lesions were induced by all test isolates. Test bacteria isolated from snails and moderately lesioned fish induced only slight lesions on test fish in comparison with the other test isolates, which demonstrated lesions within 24 h of injection. Mortalities occurred in most treatments. Often, up to 100 % of the injected fish died following injection with isolates from healthy fish and from water. All saline injected fish showed no lesions or mortalities.

Among the test catfish, ++ to ++++ grade lesions were noted within 24 h of

Table 2.5. Total bacterial counts (cfu) in fish rearing waters, sediments and golden snails (*Ampullarius* sp.) from Laguna de Bay, Pampanga and Pangasinan that contain epizootic ulcerative syndrome (EUS)-affected fish. All samples were plated on trypticase soy agar and then incubated at 25 °C for 2 d prior to counting.

Site	Culture Water	Sediments	Golden Snail
Laguna de Bay	3.90×10^3	8.30×10^5	1.80×10^6
Pampanga	3.15×10^4	2.10×10^6	8.48×10^7
Pangasinan	3.25×10^3	3.30×10^5	2.15×10^7

cfu colony forming units per ml or per gram tissue

Table 2.6. Growth and biochemical tests of representative phenotypic groups of *Aeromonas hydrophila* isolated from epizootic ulcerative syndrome (EUS)-affected catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus*) from Laguna de Bay.

Test	Code of Isolate (*) 1(A)	43B	11(C)	38(D)	62(E)	71(F)
Growth on NA	+	+	+	+	+	+
Growth in NB at 37 °C	+	+	+	+	+	+
Growth on RS Medium	G	Y	Y	Y	Y	Y
Brown pigment	-	-	-	-	-	-
Amino acids:						
(as sole source of C)						
L-arginine	+	sl +	+	+	+	+
L-alanine	+	+	+	-	+	-
L-asparagine	+	+	+	+	+	+
L-histidine	+	+	+	+	+	+
L-serine	+	+	+	-	+	-
Lipase	+	+	+	+	+	+
Caseinase	+	+	+	+	+	+
DNase	+	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+	+
Gluconate oxidase	-	-	-	-	-	-
Gelatin liquefaction	-	-	-	-	-	-
Urease	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+
Gas from glycerol	-	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+
TSI reaction:						
slant/butt	K/A	K/A	K/A	K/A	K/A	K/A
gas	-	+	-	+	+	+
H_2S	-	-	-	-	-	-
AH medium:	-	-	-	-	-	-
top/butt	K/A	K/A	K/A	K/A	K/A	K/A
motility	+	+	+	+	+	+
H_2S	-	-	-	-	-	-
indole	+	+	+	+	+	+
SIM: H_2S	+	+	+	+	+	+
motility	+	+	+	+	+	+
Simmon Citrate	+	+	+	-	-	+
Indole from tryptophan	+	+	+	+	+	+
Methyl red	+	-	+	+	+	+
Voges Proskauer	+	-	+	+	+	-
Decarboxylase						
arginine	+	+	+	+	+	+
lysine	+	+	+	+	+	-
ornithine	-	-	-	-	-	-

Table 2.6. continued

Test	Code of Isolate (*) 1(A)	43B	11(C)	38(D)	62(E)	71(F)
Acid from:						
arabinose	+	+	+	+	+	+
adonitol	-	-	-	-	-	-
cellobiose	+	+	+	+	+	+
dextrin	+	+	+	+	+	+
erythritol	-	-	-	-	-	-
esculin	+	+	+	+	+	+
fructose	+	+	+	+	+	+
dulcitol	+	+	+	+	+	+
glucose	+	+	+	+	+	+
glycerol	+	+	+	+	+	+
galactose	+	+	+	+	+	+
inositol	-	-	-	-	-	-
inulin	+	+	+	+	+	+
maltose	+	+	+	+	+	+
mannitol	+	+	+	+	+	+
mannose	+	+	+	+	+	+
lactose	+	+	+	+	+	+
rhamnose	-	-	-	-	-	-
salicin	+	+	-	-	-	-
sorbose	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
sorbitol	-	-	-	-	-	-
trehalose	+	+	+	+	+	+
xylose	-	-	-	-	-	-
Sensitivity to:						
0/129	sIS	R	sIS	R	R	R
Novobiocin	R	R	R	sIS	R	R
Hugh-Leifson test						
open	+	+	+	+	+	+
sealed	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
KCN broth	+	+	+	+	+	+
Hemolysin	ß	ß	ß	ß	ß	ß

NB Nutrient broth
RS Rimler-Shotts
G Green
K Alkaline
A Acid
S Sensitive
R Resistant

NA Nutrient agar
TSI Triple sugar iron
AH *A. hydrophila*
SIM Sulfide indole motility
KCN Potassium cyanide
sl slightly
ß Beta-hemolysis

Table 2.7. Dermal lesions and cumulative mortalities induced at 22 - 25 °C in catfish (*Clarias batrachus*) 24 - 96 h after intramuscular (IM) injection with *Aeromonas hydrophila* isolated from epizootic ulcerative syndrome (EUS)-affected snakeheads (*Ophicephalus striatus*) obtained from Laguna de Bay rearing waters, and from golden snails (*Ampullarius* sp.).

Source of Isolate	Dose Per Fish	Lesions (Cumulative Mortality) 24 h	48 h	72 h	96 h
Control	0	-* (1/2)**	-	-	-
Healthy fish skin	10^9	++++	++++ (2/2)		
Apparently healthy fish kidney	10^9	+++ (1/2)	+++ (2/2)		
Slightly lesioned fish kidney	10^{10}	+++	+++	+++	+++
Moderately lesioned fish kidney	10^9	+++	+++ (1/2)	++++ (2/2)	
Moderately lesioned fish muscle	10^9	++ (1/2)	++	++	++ (2/2)
Severely lesioned fish kidney	10^8	+++	++++	++++	++++
Severely lesioned fish muscle	10^9	++++	++++	++++	++++
Water	10^9	++++	++++	++++	++++
Golden snail	10^9	++	++	++	+

* see Table 2.3 for lesion grade criteria

** number of dead fish over total number of test fish

Table 2.8. Dermal lesions and cumulative mortalities induced at 22 - 25 °C in snakeheads (*Ophicephalus striatus*) 24 - 96 h after intramuscular (IM) injection with *Aeromonas hydrophila* isolated from epizootic ulcerative syndrome (EUS)-affected snakeheads obtained from Laguna de Bay rearing waters, and from golden snails (*Ampullarius* sp.).

Source of Isolate	Dose Per Fish	Lesion (Cumulative Mortality) 24 h	48 h	72 h	96 h
Control (n=2)	0	-*	-	-	-
Control (n=5)	0	-	-	-	-
Healthy fish skin	10^9	++(2/2)**			
Apparently healthy fish kidney	10^8	++(4/5)	++	+++	+++
Slightly lesioned fish kidney	10^{10}	++++(1/2)	++++	++++	++++
Moderately lesioned fish kidney	10^8	+(4/5)	+++	+++(5/5)	
Moderately lesioned fish muscle	10^9	-	+ (1/2)	+	+
Severely lesioned fish kidney	10^8	+++(1/5)	+++	+++(2/5)	+++
Severely lesioned fish muscle	10^7	+++(1/5)	+++	++++	++++(3/5)
Water	10^9	++++(2/2)			
Golden snail	10^9	-	+	+	+

* see Table 2.3 for lesion grade criteria

** number of dead fish over total number of test fish

bacterial injection in all test treatments but not in control fish. The most severe lesions (++++) resulted in development of deep ulcers after the necrotized musculature had sloughed (Fig. 2.1). Isolates from healthy fish, apparently healthy fish and moderately lesioned fish caused mortalities of test fish when injected intramuscularly.

The dose effect of *A. hydrophila* isolated from apparently healthy and lesioned fish in inducing dermomuscular necrosis after intramuscular injection of healthy catfish and snakeheads is shown in Table 2.9. Dermal lesions were induced only when the injected bacterial dose was at least 10^5 cfu per fish. Moreover, *A. hydrophila* was pathogenic regardless of fish species source.

The severity of dermal lesions induced by *A. hydrophila* did not vary with temperature (Table 2.10). Only isolate #53 appeared more pathogenic at the lower test temperature. In contrast, isolate #11 was pathogenic at the 10^5 dose per fish at higher temperature but not pathogenic at lower temperatures. Among the 12 isolates tested, two (#70 and #93) were non-pathogenic while the rest were virulent to the test catfish.

In all experimentally induced lesions, *A. hydrophila* was reisolated and identified as such. In addition, *A. hydrophila* was reisolated from the kidneys of experimental fish that were freshly dead or moribund.

DISCUSSION

The association of *A. hydrophila* with EUS-affected fishes has been widely reported (Roberts *et al.* 1986; Llobrera and Gacutan 1987; Torres 1990). Menasveta (1985)

Fig. 2.1. Dermomuscular necrosis/sloughing at 22 - 25 °C in catfish (*Clarias batrachus*) after intramuscular injection with *Aeromonas hydrophila*.

Table 2.9. Dermomuscular lesions and cumulative mortalities at 22 - 25 °C in catfish *(Clarias batrachus)* and snakeheads *(Ophicephalus striatus)* 96 h after intramuscular injection with different doses of *Aeromonas hydrophila* isolated from apparently healthy and epizootic ulcerative syndrome (EUS)-affected snakeheads.

Source of *A. hydrophila*	Fish Species	Dose (cfu) Per Fish	Lesion Induced	Mortality
Apparently healthy	*C. batrachus*	10^6	+++*	2/2
O. striatus		10^8	++++	2/2
	O. striatus	10^5	+++	0/5
		10^7	+++	1/5
Severely lesioned	*C. batrachus*	10^4	-	1/2
O. striatus		10^6	+++	0/2
		10^8	++++	1/2
	O. striatus	10^5	+++	0/5
		10^7	++++	4/5

cfu colony forming units
* see Table 2.3 for lesion grade criteria

Table 2.10. Effect of two rearing water temperatures on induction of dermal lesions and cumulative mortalities in catfish (*Clarias batrachus*) 24 - 96 h after intramuscular injection with two doses of various *Aeromonas hydrophila* isolates from several epizootic ulcerative syndrome (EUS)-affected snakeheads (*Ophicephalus striatus*).

Isolate	Dose Per Fish	Lesion (Cumulative Mortality) 22 - 25 °C 24 h	48 h	'72 h	96 h	30.5 - 31 °C 24 h	48 h	72 h	96 h
71	10^5	-*	-	-	-	+	-	-	-
	10^7	++ (1/2)**	- (2/2)			- (2/2)			
1	10^5	-	-	+	- (1/2)	-	-	-	-
	10^7	++ (1/2)	++ (2/2)			++ (1/2)	+++	++++ (2/2)	
38	10^5	-	-	-	-	-	-	-/+ (1/2)	-
	10^7	++++	++++	++++	++++	+++	+++	+++	+++
11	10^5	-	-	-/+	-	++	++	++	+++
	10^7	++	++ (2/2)			+++	+++ (1/2)	+++	++++
43	10^5	-	- (1/2)	-	-	-	-	-	-
	10^7	+++	+++	+++ (1/2)	-	++	++	-/+	-/+
62	10^5	-	- (1/2)	-	-	-	-	-	+ (1/2)
	10^7	+++	+++	+++	++++	+++	+++	++++	++++
50	10^5	-	-	-	- (1/2)	- (1/2)	-	-	++
	10^7	++ (1/2)	+ (2/2)			+++	+++	++++ (2/2)	
53	10^5	-	-	-	-	-	-	-	-
	10^7	++	+++	+++	+++	-	-/+	-/+	-
86	10^4	-	-	-/+	-	-	-	-	-
	10^6	+	+++	+++	+++	-	++	++	++
83	10^4	+	++	+++	+++	-	-	-	-
	10^6	+	++	+++	+++	-	+	+	+++
70	10^5	-	-	-	-	-	-	-	-
	10^7	-	-	-	-	-	-	-	-
93	10^4	-/+	-	-	-	-	-	-	-
	10^6	-/+	-	-	-	-	-	-	-
Control	0	-	-	-	-	-	- (1/2)	-	-

* see Table 2.3 for lesion grade criteria

** number of dead fish over total number of test fish

also cited this bacterium as one of the most important pathogens in EUS.

The present study confirmed the consistent presence of this bacterium in all EUS-affected fish samples. Moreover, development of bacterial populations to concentrations as high as 10^7 in severely lesioned fish was significantly correlated with dermomuscular lesions. The finding shows that high bacterial concentrations may be essential for the development of EUS-like lesions as only bacterial doses of at least 10^5 cells per fish can induce dermomuscular necrotic lesions resembling EUS.

Mortalities among experimental fish were most likely caused by bacterial infections of the kidneys as this organ showed acute septicemia and large numbers of pure cultures of the inoculated *A. hydrophila*. In contrast, the kidneys of all surviving fish that were sacrificed after 96 h were negative for the inoculated bacterium even though the lesions were positive for *A. hydrophila*.

Variations in lesion severity and mortality patterns caused by the different isolates were apparent. This variability in virulence among strains of *A. hydrophila* was reported by Torres *et al.* (1990) and Kou (1972) in infection experiments which monitored the lethality of injected *Aeromonas* isolates in grass carp and loach. Lallier *et al.* (1984) reported that the differences in observed strain virulence might be due to differences in production of extracellular products by the various strains of this pathogen. This finding was subsequently confirmed by Leaño *et al.* (1996) in experiments using extracellular proteins of *A. hydrophila* isolated from EUS-affected fish. Furthermore, the virulence of *A. hydrophila* is not correlated with siderophore secretion (Leaño *et al.* 1995).

Since *A. hydrophila* appears to be a component of the normal bacterial flora of the rearing water, pond/lake sediment, and golden snails in the areas sampled, it is probable

that these were the natural sources for infection of fish. The presence of *A. hydrophila* in freshwater bodies like Laguna de Bay has been reported earlier (Lio-Po *et al.* 1986). That *A. hydrophila* adheres to fish cells and to fish mucus and has the ability to invade epithelial cells (Krovacek *et al.* 1987) indicates that this bacterium can invade dermal tissues. This bacterium is pathogenic to: (1) milkfish (*Chanos chanos*) fingerlings by bath after scale removal (Lio-Po and Duremdez-Fernandez 1986); (2) catfish (*C. batrachus*) when injected intraperitoneally (Supriyadi 1986); and (3) channel *catfish (Ictalurus punctatus*) after mechanical abrasion (Ventura and Grizzle 1987). *A. hydrophila* was associated with superficial infections of Indian carp (*Catla catla*) and can lead to ulcer development as reported in India (Karunasagar *et al.* 1986). Huizinga *et al.* (1979) described the pathology of dermal necrosis, involving the underlying musculature in largemouth bass (*Micropterus salmoides*) following infection with *A. hydrophila.*

The present study demonstrated the potential of *A. hydrophila* to produce EUS-like lesions in snakehead and catfish. The rapid onset and development of dermomuscular necrotic lesions (within 24 h of inoculation of a pure culture of *A. hydrophila*) indicated an acute effect on the fish. The subsequent appearance of deep ulcers (starting at 72 h post-injection) that are similar to the EUS lesions observed in natural outbreaks of this disease might represent the chronic phase of the disease. The induced ulcerative lesions localize within a 2 - 4 cm diameter at the site of intramuscular injection. Krovacek (1989) experimentally induced similar lesions in rainbow trout (*Oncorhyncus mykiss*) when 10^7 cells of *A. hydrophila* were injected intramuscularly. It is also interesting to note that blanching of injected integument, observed at an early stage of dermonecrotic damage in this study, was also reported by Krovacek (1989) as an effect of intramuscular injection of

extracellular proteins (ECP) in rainbow trout (*O. mykiss*). Thus, one can deduce the possible role of *A. hydrophila* ECP in initiating EUS-like lesions or in destroying the host tissue and enhancing the spread of *A. hydrophila* infection in vivo.

A. hydrophila infections were reported in milkfish in association with stressful conditions such as transport (Lio-Po *et al.* 1986). Subsequent experiments by Lio-Po and Duremdez-Fernandez (1986) showed that scale removal induced septicemic infections in healthy milkfish fingerlings continuously exposed to *A. hydrophila* in the rearing water. Ventura and Grizzle (1987) reported that *A. hydrophila* gained access to the internal organs of channel catfish through the digestive tract or uninjured skin under crowded conditions (13.1 kg of fish per m^3 rearing water) and high water temperature (24 °C).

The data presented here suggest that certain phenotypes of *A. hydrophila* can induce EUS-like lesions in both snakeheads and catfish when injected subdermally in dosages of 10^5 or more cells. The lesions induced in this manner are very similar to the EUS lesions observed in naturally infected catfish and snakeheads. However, considering the large dose needed to induce the lesion and the development of lesions at both low and high test temperatures suggest that the bacterium (although possibly involved in the final development of the lesions) is not the primary cause of EUS. If *A. hydrophila* is one of the causative agents of EUS, as appears possible, the means by which this pathogen initiates the infection is unclear. It seems probable that the integument of these fish must be injured, in order to allow entrance of the *A. hydrophila* cells, which subsequently produce EUS-like lesions.

Processes such as scale removal and scrapes, parasite attachment, and bites from predatory fish may produce injuries to the integument. It should be noted that at all

sampling sites of this study, the golden snail was present. These snails were introduced into the Philippines in the early 1980's and the first EUS outbreak occurred in late 1985. Many fish farmers have observed that these snails attach to the fish's integument, leaving superficial lesions when they drop off. Furthermore, injury to the integument among snakehead and catfish can result from the cannibalistic attack of one fish upon another under conditions of starvation and increased stocking density.

CHAPTER 3

EXPERIMENTAL INDUCTION OF EUS-LIKE LESIONS IN CATFISH (*CLARIAS BATRACHUS*) AND SNAKEHEADS (*OPHICEPHALUS STRIATUS*) WITH *AEROMONAS HYDROPHILA*, *AQUASPIRILLUM* SP., *PSEUDOMONAS* SP., AND *STREPTOCOCCUS* SP.

INTRODUCTION

Outbreaks of the EUS among catfish (*C. batrachus*) and snakeheads (*O. striatus*) in both wild and cultured fish in South and Southeast Asia occur annually (Lilley *et al.* 1992). The Philippines and Thailand are among the Asian countries seriously affected by the EUS outbreaks. The pathogenicity of *A. hydrophila* is consistently associated with EUS-affected fishes in these countries and its association with fish pathology is strongly indicated by the data in Chapter 2. However, the role of other bacteria associated with the EUS lesion have not been established. Consequently, experiments were undertaken to characterize other EUS-associated bacteria and to test their virulence to healthy catfish and snakeheads.

MATERIALS AND METHODS

Test Bacteria

Isolates of *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp. were obtained from EUS-affected snakeheads, 20 - 30 cm long, from two fish farms located in Samutsonkram and in Suphanburi, Thailand. Nine fish from each locality were sampled. Kidney, spleen and liver fragments from each fish were crushed together, brain and gills were processed individually, and subcutaneous tissue beneath the margin of the ulcers was sampled by scraping. Materials collected from three similarly lesioned fish were pooled for inoculation onto TSA, TSA containing 10 % sterile horse serum, and Anacker and Ordal agar (AOA).

Isolates were purified and key characteristics were determined using API 50, API 20 E or NE (API, La Balme-les-Grottes, France) and conventional tube tests. DR. P. RICHARD (Laboratory of Enterobacteriaceae, Pasteur Institute, Paris) confirmed some of the bacterial identifications. The *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp. isolates were used for subsequent pathogenicity experiments.

At the same time, *A. hydrophila* was isolated from EUS-affected snakeheads collected from Laguna de Bay, Philippines. Bacteria were isolated from muscle lesions or kidney separately using Rimler-Shotts medium and TSA (BBL). Bacterial colonies were purified on Difco nutrient agar (NA) and characterized by Gram staining, biochemical reactions and growth in various media. Final identification was based on the method of Collins and Lyne (1984) and the identification guide of Popoff (1984) for *Aeromonas*.

Pathogenicity Experiments

Healthy catfish (15 - 25 g) and snakeheads (15 - 25 g) were collected from rivers in Iloilo, Philippines, where EUS is not known to be present. These two species of fishes were chosen as test fishes because they were the most economically important species affected by EUS in the Philippines. The fishes were transported to SEAFDEC and stocked separately in 1000-liter fiberglass tanks of UV-treated water. These fishes were acclimated for at least 1 week prior to infection experiments.

Infection experiments were conducted in disinfected 8-liter aquaria, filled with UV-treated freshwater and aerated. Two catfish or two snakeheads were stocked per aquarium. Two dilution-treatments with three replicates were used for each bacterial isolate. Rearing water temperature was maintained at 22 - 25 °C.

All test bacteria were passaged twice in healthy catfish prior to infection experiments. Pure cultures of *A. hydrophila*, *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp., were then subcultured on TSA and the colonies harvested after 24 h incubation, except for *Pseudomonas* sp. which was harvested after 48 h. Harvested bacteria were diluted with sterile NSS to densities of 10^{10} and 10^{6} cfu per ml for *A. hydrophila* and the *Aquaspirillum* sp. and 10^{9} and 10^{5} cfu per ml for the *Pseudomonas* sp. and *Streptococcus* sp., respectively. Test fish were injected with 0.1 ml of the test suspension intramuscularly. Control fish were inoculated with sterile NSS.

Development of lesions and fish mortality were monitored daily until 96 h post-injection. Criteria for grading lesion development in experimental fishes were as in Table 2.3.

Bacterial reisolations were made from muscle/lesion and kidney of the experimentally infected fishes according to procedures described in Chapter 2. Identification of the bacterial isolates was confirmed by biochemical tests.

Statistical Test

Fish mortality data were processed by a Repeated Measures Analysis of Variance.

RESULTS

From EUS-affected fish sampled in Thailand, 56 bacterial isolates were obtained and studied. Motile *Aeromonas* was the predominant isolate. *Streptococcus* sp. was isolated from the brain of one fish. *Aquaspirillum* sp. was isolated from skin lesions of fish from Samutsonkram farm and from the brain of EUS fish in Suphanburi farm. *Pseudomonas* sp. was isolated from the skin samples of EUS fish with less severe lesions collected in Samutsonkram. This fastidious isolate required 72 - 96 h at 37 °C to produce pinpoint colonies. *A. hydrophila* was the predominant and consistently isolated bacterium from EUS skin lesions of snakeheads in the Laguna de Bay, Philippines, in an earlier study (Chapter 2). Results of the bacterial identification tests are summarized in Table 3.1.

Intramuscular injection of healthy catfish and snakeheads with *A. hydrophila* induced slight to severe dermomuscular lesions as early as 24 h post-injection as described in Table 2.3. Control fish did not develop any lesions.

Table 3.1. Growth, biochemical and Gram stain characteristics of bacterial isolates: *A. hydrophila (Ah), Aquaspirillum sp. (Aq), Pseudomonas sp. (Ps), Streptococcus sp. (St) isolated* from EUS-affected snakeheads *(Ophicephalus striatus) from the* Philippines (Ah) and from Thailand (Aq, Ps and St).

Tests	Ah	Aq	Ps	St
Gram staining	-	-	-	+
Motility	+	+	+	-
Pigment	-	-	-	-
Oxidase	+	+	+	-
Catalase	NT	+	+	-
H_2S	+	-	NT	-
Nitrate reduction	+	+	+	NT
Simmon's citrate	+	-	-	NT
Gelatin liquefaction	-	-	-	NT
Urease	-	+	-	NT
Indole	+	-	-	NT
Voges Proskauer	+	-	NT	NT
Decarboxylase:				
Ornithine	-	-	NT	NT
Lysine	+	-	NT	NT
Arginine	+	-	-	-
Hugh-Leifson test:				
open	+	-	-	NT
sealed	+	-	-	NT
TSIA				
slant/butt	K/A	K/K	K/K	A/A
gas	-	-	-	-
H_2S	-	-	-	-
Growth at:				
37 °C	+	NT	+	+
41 °C	NT	-	+	NT
0% NaCl	NT	+	NT	+
2%	NT	+	NT	+
4%	NT	-	NT	-
6.50%	NT	NT	NT	-
7.50%	NT	NT	NT	-
Growth on McConkey agar	+	NT	+	-

Table 3.1. continued

Tests	Ah	Aq	Ps	St
Agglutination:				
in acriflavine	NT	NT	NT	+
at 100 °C	NT	NT	NT	+
Hemolysin	ß	NT	NT	ß
Sensitivity to:				
0/129 (150 µg)	sl S	R	NT	NT
Novobiocin	R	NT	NT	NT
Acid from:				
Adonitol	-	NT	NT	-
Arabinose	+	-	-	-
Cellobiose	+	NT	NT	+
Erythritol	-	NT	NT	-
Esculin	+	-	-	+
Acid from:				
Fructose	+	NT	NT	+
Galactose	+	NT	NT	+
Glucose	+	-	-	+
Glycerol	+	-	NT	-
Inositol	-	NT	NT	-
Inulin	+	NT	NT	-
Lactose	+	-	NT	-
Malate	NT	-	-	NT
Maltose	+	+	+	+
Mannose	+	-	-	+
Mannitol	+	-	-	-
Rhamnose	-	NT	NT	-
Saccharose	NT	+	NT	+
Salicin	-	NT	NT	+
Sorbitol	-	NT	NT	-
Trehalose	+	-	NT	+
Xylose	-	-	NT	-

NT not tested
K alkaline
A acid
TSIA Triple Sugar Iron Agar
ß beta-hemolysin
S sensitive
R resistant
sl slightly

In tests with catfish, *Aquaspirillum* sp., *Pseudomonas* sp., *Streptococcus* sp., and *A. hydrophila* induced slight (+), moderate (++ to +++) and severe (++++) dermomuscular necrotic lesions, respectively, 1 to 4 d after IM injection. The dermomuscular lesions (++) produced by *Streptococcus* sp., persisted for 72 h post-injection while those induced by *Aquaspirillum* sp. and *Pseudomonas* sp. showed initial healing 48 and 72 h, respectively.

A cumulative mortality of 50 % was observed among test catfish injected with the higher dose of *A. hydrophila*. One of six fish in the lower dose *Streptococcus* treatment died during the 96 h post-injection period (Table 3.2). The observed mortalities in the four bacterial treatments, however, were not statistically significant ($p<0.05$) from those of the controls.

Among injected snakeheads, only *A. hydrophila* induced severe lesions (++++). *Aquaspirillum* sp. and *Pseudomonas* sp. did not produce any dermal lesions while *Streptococcus* sp. induced slight lesions (+) at 48 h post-injection which healed rapidly. Mortality occurred in groups injected with lower doses of *A. hydrophila* (1/6), and low levels of *Aquaspirillum* (2/6) and *Pseudomonas* (2/6). No mortality occurred among control fishes and the few fish that died in the groups injected with lower doses of *Streptococcus* or *Pseudomonas* were not significantly different from the deaths of the controls (Table 3.2).

DISCUSSION

The data obtained in this chapter showed that catfish and snakeheads differed in their susceptibility to four bacterial isolates (Table 3.2).

Table 3.2. Lesions and cumulative mortalities in catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus*) at 22 - 25 °C, 24 - 96 h after intramuscular injection of six of each fish species with two doses of *Aeromonas hydrophila, Aquaspirillum sp., Pseudomonas sp.*, and *Streptococcus* sp.

Treatment	Dose (cfu) Per Fish	Lesion (Mortalities) on Day Post-exposure 1	2	3	4
Catfish					
Control	0	-*	-	-	-
A. hydrophila	10^9	+++(1/6)	+++(2/6)	+++(3/6)	++++(3/6)
	10^5	+	+	+/-	+/-
Aquaspirillum sp.	10^9	+	+/-	+/-	+/-
	10^5	-	-	-	-
Pseudomonas sp.	10^8	+	+	+/-	+/-
	10^4	-	-	-	-
Streptococcus sp.	10^8	++	++	++	+
	10^4	-	-	-(1/6)	-(1/6)
Snakeheads					
Control	0	-	-	-	-
A. hydrophila	10^9	+++	+++	+++	++++
	10^5	-	-	-	-(1/6)
Aquaspirillum sp.	10^9	-	-	-	-
	10^5	-	-	-(1/6)	-(2/6)
Pseudomonas sp.	10^8	-	-	-	-
	10^4	-	-(1/6)	-(2/6)	-(2/6)
Streptococcus sp.	10^8	-	+	-	-
	10^4	-	-	-	-

cfu colony forming units

* see Table 2.3 for lesion grade criteria

Number in ( ) cumulative mortality/total fish stocked

A. hydrophila induced severe dermomuscular necrotic lesions in both catfish and snakeheads. *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp. were slightly pathogenic to catfish, inducing dermomuscular lesions while in snakeheads only *Streptococcus* sp. was slightly pathogenic. The mortalities in catfish and snakeheads subjected to low doses of *Aquaspirillum* sp., *Pseudomonas* sp. and *Streptococcus* sp. may be experimental artifacts because the high doses produced no deaths. Isolation of other bacteria from EUS-lesioned fish, such as *Pseudomonas* sp. and *Streptococcus* sp., have also been reported (Menasveta 1985; Boonyaratpalin 1989; Pal and Pradhan 1990; Subasinghe *et al.* 1990). *Aquaspirillum*, on the other hand, has not been reported previously to be associated with fish diseases, and the present study is the first reported evidence of its association with diseased fish. This bacterium generally occurs in stagnant, eutrophic pondwaters (Krieg 1984).

Previous studies demonstrated the pathogenicity of *A. hydrophila* for EUS-susceptible fishes such as catfish and snakeheads (Angka 1990; Chapter 2; Pathiratne *et al.* 1994; Supriyadi 1986) and for climbing perch, *Anabas testudineus* (Pal and Pradhan 1990). The latter workers used individual inoculations with *A. hydrophila* and two species of *Pseudomonas*. Severest lesions occurred in fish inoculated with *A. hydrophila*. A combination of *A. hydrophila*, *Pseudomonas* and *Micrococcus* inoculated into *Anabas* induced deep ulcers that were more severe than those produced when these bacteria were inoculated singly. Pathiratne *et al.* (1994) observed necrotic and deep ulcers but no mortalities among snakeheads after intramuscular injection with *A. hydrophila*.

In the present study among the four EUS-associated bacteria tested, *A. hydrophila* was the most pathogenic, inducing ulcerative dermal lesions similar to those observed

among EUS-affected fishes. The rapid onset of lesions within 24 h of intramuscular injection indicated an acute effect. Subsequent studies showed that in catfish challenged with *A. hydrophila*, the lesion onset was dose related (Chapter 4). In studies with channel catfish, *Ictalurus punctatus*, Grizzle and Kiryu (1993) concluded that *A. hydrophila* infections are of three categories: (1) motile aeromonad septicemia (MAS; systemic infection and signs of disease), (2) cutaneous (infection limited to skin and underlying muscle), and (3) latent (systemic infection but no external signs of disease). Apparently, in intramuscularly injected catfish and snakeheads, the virulence of *A. hydrophila* is of the cutaneous type. The dermatotoxic pathology induced by *A. hydrophila* was probably a consequence of the elaboration of a dermonecrotic factor which has been shown experimentally to induce cutaneous lesions in test rabbits and rainbow trout (Krovacek *et al.* 1987; Lallier *et al.* 1984; Olivier *et al.* 1981; Santos *et al.* 1992). Crude extracellular proteins from different strains of *A. hydrophila*, however, induced different degrees of dermomuscular necrotic lesions in catfish (Leaño *et al.* 1996).

The mortalities observed among some of the catfish injected with *A. hydrophila* were likely the result of the progression of the infection to the septicemic form. Previous work has shown that mortality rates caused by *A. hydrophila* in catfish varies with the *A. hydrophila* strain used (Chapter 2; Leaño *et al.* 1996). In studies where *A. hydrophila* was injected intraperitoneally, the MAS type of pathology developed in goldfish, *Carassius auratus* (Brenden and Huizinga 1986), milkfish, *Chanos chanos* (Lio-Po and Duremdez-Fernandez 1986), catfish, *C. batrachus* (Supriyadi 1986; Angka 1990), and grass carp, *C. idella* (Torres *et al.* 1990).

My finding that *A. hydrophila* induces cutaneous lesions with or without systemic involvement in intramuscularly-injected catfish and snakeheads is in agreement with the results of Grizzle and Kiryu (1993) on channel catfish experimentally infected with *A. hydrophila*. My experiments further confirmed that among the four EUS-associated bacteria tested, only *A. hydrophila* was highly pathogenic to both test fish species, inducing ++++ lesions. *Pseudomonas* sp. was slightly pathogenic (+) to catfish but not to snakeheads. *Aquaspirillum* sp. and *Streptococcus* sp. were both slightly pathogenic (+ and ++) to catfish but not to snakeheads.

CHAPTER 4

EXPERIMENTS ON VIRULENT DOSE AND PORTALS OF ENTRY OF *AEROMONAS HYDROPHILA* IN CATFISH (*CLARIAS BATRACHUS*)

INTRODUCTION

The occurrence of the EUS among catfish (*C. batrachus*) and snakeheads (*O. striatus*) has been observed annually in the Philippines and many countries in Asia. In the Philippines, EUS has been associated with the bacterium, *A. hydrophila* (Chapter 2; Llobrera and Gacutan 1987). In other countries, the association of *A. hydrophila* with EUS-affected fishes has also been reported (Boonyaratpalin 1987; Roberts *et al.* 1986; Subasinghe *et al.* 1990; Torres *et al.* 1990). Recent studies demonstrated the consistent association of this bacterial pathogen with EUS-affected fishes and their rearing waters as well as with the laboratory inducement of dermomuscular lesions in intramuscularly injected healthy catfish and snakeheads (Chapter 2). However, the minimum virulent dose and the various possible portals of entry for the bacterial pathogen in catfish have not been established. Moreover, fish farmers claim that the golden snails which are abundant in the catfish rearing waters are often seen attached to the catfish and possibly injure the fish. Thus, the objective of this study was to establish the minimum dose of *A. hydrophila*

that would induce EUS-like lesions and to test various portals of entry of the bacterial pathogen in catfish.

MATERIALS AND METHODS

Experimental Fish and Snails

Experiments used 8-liter aquaria disinfected with 70 % ethanol and filled with 5 liters of UV-sterilized freshwater at SEAFDEC, Iloilo. Catfish, 20 - 30 g, collected from Iloilo, were stocked at two per aquarium, while golden snails were stocked at 10 per aquarium. Each treatment was replicated three times. Water temperature was maintained at 22 - 25 °C with aeration. The fish were not fed during the experiments.

Test Bacteria

Pure cultures of two isolates, #1 and #11, of *A. hydrophila* isolated from kidneys of normal and EUS-affected snakeheads, respectively, were subcultured in TSA plates and harvested after 24 h incubation by gently scraping colonies from the agar surface, using sterile inoculating loop. Harvested bacteria were then diluted with sterile NSS to the desired densities for each treatment.

Dose Response Experiment

For virulent dose determination, 0.1 ml of bacterial suspensions of *A. hydrophila* were injected intramuscularly at the epaxial muscles into catfish at doses of 10^8, 10^6, 10^5,

10^4, 10^3, 10^2, and 10^1 cfu per ml. Control fish were injected with 0.1 ml sterile NSS. The bacterial doses were determined by plate count after serial dilution of the harvested bacteria.

Portals of Entry Experiment

For the portal of entry experiments, *A. hydrophila* was inoculated into the rearing water to bacterial densities of 10^6 and 10^8 cfu per ml. Treatments included catfish injured by (1) a muscle cut (MC) 1 cm in length and 0.5 cm in depth using a sterile scalpel blade, (2) dermal scraping (DS) achieved by rubbing the dorsal/blunt edge of a scalpel blade over a 1 cm^2 surface area of skin, (3) fish bite (FB) by pressing the open mouth of a larger snakehead along the dorsal side of the catfish torso ensuring that the fish teeth of the snakehead pierced the skin of the test catfish and (4) cohabitation with golden snail (CS). Untreated fish served as controls. In other treatments, bacterial inocula of 10^5 and 10^7 cfu per fish were given by (1) intramuscular injection (IM), (2) feeding after injecting the bacterial inoculum into the center of a morsel of fish food muscle (Or), (3) gastric lavage by introducing the bacterial inoculum via the mouth of the catfish using a syringe (GL). Control fish for each treatment were inoculated with sterile NSS.

The fish were treated with bacteria at the time they were stocked except for the cohabitation challenge involving golden snails where the aquarium water was inoculated 48 h after stocking.

Monitoring and Bacterial Reisolation

Mortalities and development of lesions were monitored daily for 9 d for the portals of entry experiment and for 16 d for the dose-response experiment. The severity of lesion development was recorded according to the criteria in Table 2.3.

Inoculated *A. hydrophila* was reisolated from muscles/lesions and kidneys of laboratory infected fish. Bacterial isolates were purified on TSA, characterized, and confirmed as *A. hydrophila* by biochemical tests including decarboxylase, oxidase, oxidation-fermentation of glucose, nitrate reduction, and inoculation of *A. hydrophila* (AH) medium.

Statistical Tests

Mortality data were transformed to arcsine values and subjected to a two-way analysis of variance. Subsequently, the Duncan's multiple range tests was applied to establish statistical differences among means.

RESULTS

Dermal lesions were induced by the # 11 *A. hydrophila* isolate only in catfish injected with 10^5 and 10^7 cfu per fish (Table 4.1). Lesions induced by the 10^7 bacterial dose were most severe, with necrosis of the underlying injected musculature. The localized lesion was excavated by d 2 post-injection. At the 10^5 dose treatment, eroded lesions developed but they reversed in intensity by d 7. Although apparent healing lesions were observed by d 9, complete healing did not develop through d 16 post-injection.

Table 4.1. Lesion development and cumulative mortalities at 22 - 25 °C in catfish (*Clarias batrachus*) injected intramuscularly with various concentrations of *Aeromonas hydrophila* isolates obtained from healthy (#1) and from epizootic ulcerative syndrome (EUS)-affected (#11) snakeheads (*Ophicephalus striatus*).

Dose (cfu) Per Fish	Isolate No.	Lesions (Mortalities) on Day Post-exposure									
		1	2	3	4	5	6	7	8	9	10
Control	NSS	-*	-	-	-	-	-	-	-	-	-
	NSS	-	-	-	-	-	-	-	-	-	-
10^7	1	+++	++++(1/6)	+++(2/6)**	++++**	++++**	++++**	++++(3/6)**	++++(5/6)**	++++**	++++(6/6)**
	11	+++(1/6)	++++	++++(3/6)**	++++(4/6)**	++++(5/6)**	++++**	++++(6/6)**			
10^5	1	+++(1/6)	+++	+++	+++	+++	+++	+++	++	++	+/-
	11	+++	+++	+++	+++	+++	+++	++	+	+/-	+/-
10^4	1	-	-(1/6)	-	-	-	-	-	-	-	-
	11	-(1/6)	-	-	-	-	-	-	-	-	-
10^3	1	-	-	-	-	-	-	-	-	-	-
	11	-(1/6)	-	-	-	-	-	-	-	-	-
10^2	1	-	-	-	-	-	-	-	-	-	-
	11	-(1/6)	-	-	-	-	-	-	-	-	-
10	1	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-	-

cfu colony forming units
NSS 0.85 % NaCl solution
Number in ( ) number dead/number fish tested

* see Table 2.3 for lesion grade criteria
** highly significant differences in cumulative mortality among treatments and controls ($p<0.01$)

Significant cumulative mortalities ($p<0.01$) in the 10^7 cfu treated fish started to occur 24 h after IM injection and total mortality occurred by d 7. At dose treatments lower than 10^5, mortalities were not significant in test fish (Fig. 4.1).

Similar observations were apparent in catfish injected with # 1 *A. hydrophila*, isolated from a healthy snakehead (Table 4.2). Lesions developed only in the 10^5 and 10^7 cfu per fish treatments. Completely healed lesions were not apparent at d 16 after injection. Mortalities observed at the 10^7 cfu per fish treatment were significantly different ($p<0.01$) from those of the lower dose treatments. In general, mortalities occurred at a lesser rate at the 10^7 cfu per fish treatment in comparison with mortalities caused by the *A. hydrophila* isolate (# 11) from EUS-affected snakehead. Cumulative mortalities differed significantly on d 5, 6 and 7 from the controls (Fig. 4.1).

On culturing the test fish for bacteria, abundant quantities of pure cultures of *A. hydrophila* were recovered from lesions and kidneys of the test fish in the 10^5 and 10^7 cfu per fish treatments while at lower dose treatments, mixed colonies were recovered. In tests on portals of entry of *A. hydrophila*, only the intramuscular injection of 10^5 and 10^7 per fish treatments induced dermal lesions in the test catfish. Lesion severity was directly related to dose and post-exposure period. Mortalities observed were likewise significantly higher ($p<0.05$) than those of other treatments. The test bacteria were reisolated in pure cultures only in the IM treated fish.

Fig. 4.1. Cumulative mortalities at 22 - 25 °C of catfish (*Clarias batrachus*) after intramuscular injection with 10^7 and 10^5 cfu per fish of *Aeromonas hydrophila*.

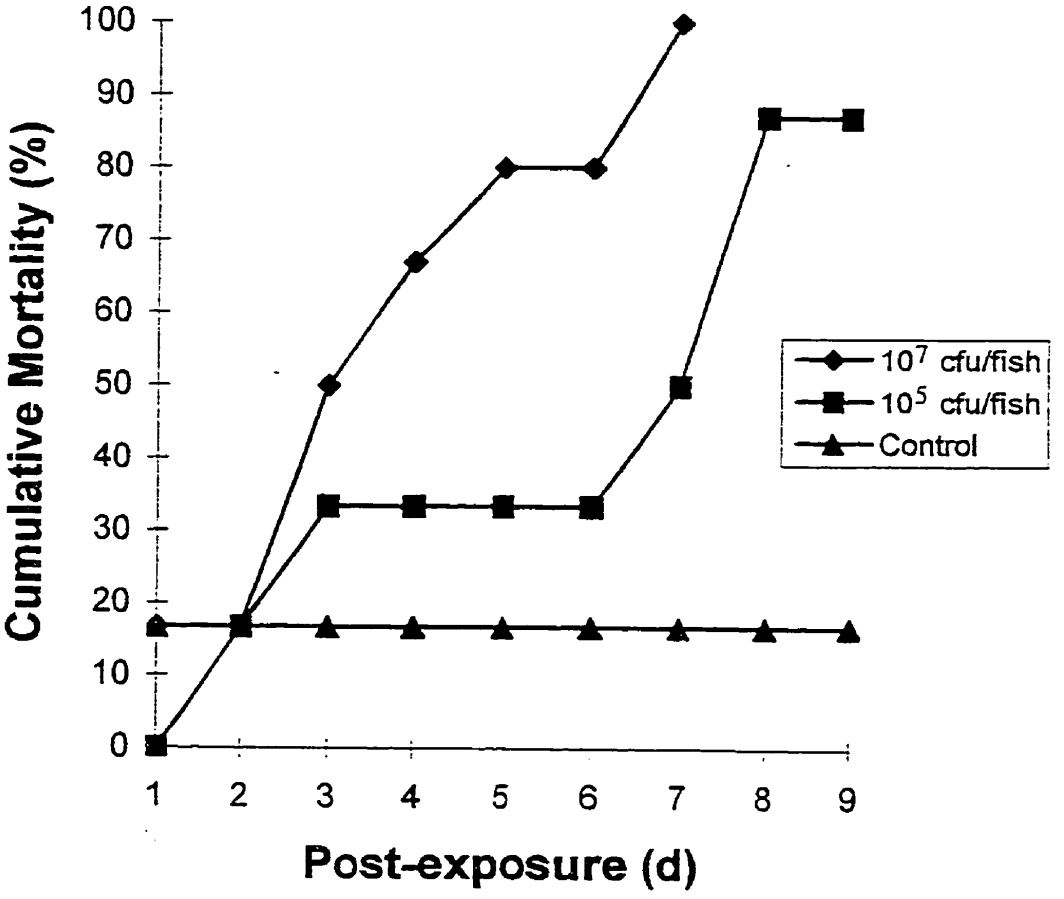

Cumulative Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
Post-exposure (d)
10^7 cfu/fish
10^5 cfu/fish
Control

Table 4.2. Lesion development and cumulative deaths at 22 - 25 °C in catfish (*Clarias batrachus*) exposed to *Aeromonas hydrophila* isolate #11 using various portals of entry.

Treatment	Lesions (Mortalities) on Days Post-exposure								
	1	2	3	4	5	6	7	8	9
IM Control	-*(1/6)	-	-	-	-	-	-	-	-
IM 10^5	+(1/6)	++	++	+++	+++	+++	+++	+++	+++(2/6)
IM 10^7	+(1/6)	+++(3/6)	+++(4/6)	++++	++++(6/6)				
Or Control	-	-	-	-	-	-	-	-	-
Or 10^5	-	-	-	-	-	-	-	-	-
Or 10^7	-	-	-	-	-	-	-	-	-
GL Control	-	-	-	-	-	-	-	-	-
GL 10^5	-	-	-	-	-	-	-	-	-
GL 10^7	-	-	-	-	-	-	-	-	-
DC Control	-	-	-	-	-	-	-	-	-
DC 10^5	-	-	-	-	-	-	-	-	-
DC 10^7	-	-	-	-	-	-	-	-	-
DS Control	-	-	-	-	-	-	-	-	-
DS 10^5	-	-	-(1/6)	-	-	-	-	-	-
DS 10^7	-	-	-	-	-	-	-	-	-
FB Control	-	-(2/6)	-	-	-	-	-	-	-
FB 10^5	-	-	-	-(1/6)	-	-	-	-	-
FB 10^7	-(1/6)	-	-	-	-	-	-	-	-
CS Control	-	-	-	-	-	-	-	-	-
CS 10^5	-	-	-	-	-(1/6)	-	-(2/6)	-	-
CS 10^7	-	-(1/6)	-	-	-	-	-	-	-
IMM Control	-	-	-	-	-	-	-	-	-
IMM 10^5	-	-	-(1/6)	-	-	-	-	-	-
IMM 10^7	-	-	-	-(1/6)	-	-	-	-	-

IM	intramuscular injection	Or	oral/food
DC	dermal cut	GG	gastric lavage
DS	dermal scraping	CS	cohabitation with golden snail
FB	fish bite	IMM	immersion
*	see Table 2.3 for lesion criteria		
Control	no bacterial inoculum		
Number in ( )	number dead/number fish tested		

DISCUSSION

Results of the present study indicate that a dose of at least 10^5 cfu of bacteria per fish injected intramuscularly was necessary to induce dermal lesions in catfish. This confirms results of previous infection experiments in both snakehead and catfish (Chapters 2 and 3). This dose was sufficient to cause septicemic infection as indicated by the positive isolation of the test bacterium from the test fish kidneys. Septicemia eventually resulted in the death of test catfish, particularly, in the 10^7 cfu per fish treatment.

Systemic infections of *A. hydrophila* in goldfish, *C. auratus*, resulted in infection of the hemopoietic system, degeneration of the liver and mild necrosis of the kidney (Brenden and Huizinga 1986). Other studies on *A. hydrophila* injected intraperitoneally at 10^5 cfu per fish into *C. batrachus* likewise yielded significant mortalities (Angka 1990). Supriyadi (1986) showed that *A. hydrophila* was most virulent to catfish (*C. batrachus*), slightly virulent to giant gouramy (*Osphronemus gouramy*) but nonvirulent to common carp (*C. carpio*), at the 10^5 cells per fish dose.

The fact that *A. hydrophila*, after introduction to test catfish by varying test portals of entry, failed to induce dermal lesions except through intramuscular injection indicates that the mere presence of 10^5 *A. hydrophila* cfu per ml in rearing waters is not sufficient to cause muscular lesions or septicemic infection of exposed catfish. Studies have shown that pond water columns inhabited by catfish and snakeheads can have up to 10^7 bacteria per ml (Chapter 2). Hence, it is hypothesized that only fish with dermal injuries are susceptible to infection. It appears that in catfish, a build-up of this bacterial pathogen to the virulent concentration of at least 10^5 cells is needed to induce EUS-like lesions and

mortalities. It is also possible that the development of lesions may be facilitated by benthic conditions. The walking catfish is normally a pond/lake bottom dweller, where the bacterial population is 10 to 20 times greater than that in water (Lewis and Bender 1961). Moreover, catfish and snakeheads are cannibalistic in nature and therefore likely to injure other fish species.

Similarly, in studies on pathogenicity of *A. hydrophila* to Nile Tilapia (*Sarotherodon niloticus*) in Egypt, infections were experimentally transmitted by intraperitoneal and intramuscular injections but not by scarification of the epidermis (Amin *et al.* 1985). *A. hydrophila*, likewise, induced highly significant mortalities after intraperitoneal injections at 10^6 cfu per 100 g fish dose in Tilapia (*Oreochromis mossambicus*) but not in immersion exposure of descaled fish (Lio-Po *et al.* 1987). In contrast, in milkfish (*C. chanos*) fingerlings *A. hydrophila* proved pathogenic after immersion exposure of descaled fish to 10^5 cfu per ml dose (Lio-Po and Duremdez-Fernandez 1986).

Cohabitation experiments showed that the fish farmers' belief (pers. comm.) that the golden snails thriving in the fish rearing waters of lakes and freshwater ponds can injure the fish and predispose them to develop EUS-like lesions appears to be unfounded. This polyculture situation did not provoke dermal injury among catfish that could serve as portals of entry for the pure culture of *A. hydrophila* added to the waters during the experiment, notwithstanding the indigenous bacterial load of the snails (Chapter 2).

A. hydrophila is considered an opportunistic pathogen of fish. Studies on channel catfish have demonstrated the relationship of water quality and infectious disease development (Plumb 1984). Thus, the influence of environmental factors causing stress

on fish that subsequently leads to *Aeromonas* infections must not be ruled out. In addition, the probable presence of a primary pathogen should be investigated.

CHAPTER 5

ESTABLISHMENT OF CELL LINES FROM CATFISH (*CLARIAS BATRACHUS*) AND SNAKEHEADS (*OPHICEPHALUS STRIATUS*)

INTRODUCTION

Chapters 2, 3 and 4 demonstrated the ability of *A. hydrophila* to induce muscular necrosis in catfish and snakeheads. However, the data of Chapters 2, 3 and 4 do not unequivocally support the hypothesis that *A. hydrophila* is the primary causal agent of EUS. Hence, it was necessary to determine if other microbial pathogens act as the causative agent of EUS.

Frerichs *et al.* (1986), after their investigation of EUS outbreaks in Thailand and Burma, reported the isolation of a rhabdovirus from snakeheads and freshwater eels (*Fluta alba*). Although, Noga and Hartmann (1981) and Frerichs *et al.* (1986) reported the establishment of cell lines from adult catfish gill, gonad and kidney tissue and from snakehead fry fins, respectively, these cell lines are not readily available. It was therefore necessary to establish cell cultures from EUS-susceptible fishes to investigate the role of viruses in the development of EUS in these fish.

The objective of this study was to establish cell lines from indigenous catfish and snakeheads and to determine if these cell lines were susceptible to the 91-97 virus isolated from an EUS-lesioned fish.

MATERIALS AND METHODS

Tissue Processing and Primary Cell Culture

Catfish and snakeheads, whole fry and fingerlings (2 - 3 g) were externally disinfected with 70 % ethanol, followed by 3 mg per L iodophore solution. Whole fry were then rinsed with sterile NSS before being aseptically cut into fragments (about 1 mm^3). Brain, fins, gonad, heart, kidney, liver, skin and spleen tissues were removed from each fingerling. Each organ was individually rinsed aseptically three times in Pert dishes containing Eagles balanced salt solution (EBSS) plus an antibiotic-antimycotic mixture (200 I.U. penicillin G sodium, 200 ug streptomycin sulfate, 50 ug amphotericin B per ml medium). The organs were then minced thoroughly using sterile scissors. These were subjected to one of four treatments: Method A: explants were placed onto the surface of 25-cm^2 Primaria flasks (Falcon) and allowed to attach for 1 h before the addition of Leibovitz's medium (L-15) supplemented with 15 % fetal bovine serum (FBS) (L15-15); Method B: explants were inoculated into 25-cm^2 Primaria flasks (Falcon) already containing L15-15 (R. Hedrick, Univ. Calif.-Davis, pers. comm.); Method C: tissue fragments were passed through a stainless steel sieve with the aid of a syringe plunger into a Petri dish containing L15-15 medium. Method D: tissue fragments were digested

with 0.5 % trypsin-EDTA slowly agitated with a magnetic stirrer for 1 h at 25 °C (Chen *et al.* 1983). For methods C and D, aliquots of the organ tissue were transferred to 25-cm^2 Primaria tissue culture flasks (Falcon) containing about 12 ml of L15-15.

At least three flasks were inoculated for each organ tissue. All flasks were incubated at 25 °C. Every 2 d, half of the medium in each flask was replaced with fresh culture medium. The flasks were also examined microscopically daily for monolayer cell outgrowth around the tissue fragments for 4 weeks. All media were supplemented with an antibiotic-antimycotic mixture (100 I.U. penicillin G sodium, 100 ug streptomycin sulfate, and 25 ug amphotericin B per ml medium).

Cell Passages

Cells in flasks showing moderate cell outgrowths were split using trypsin-EDTA (0.5 % Trypsin-5.3 mM EDTA, Gibco). The cell sheets were rinsed once with one ml trypsin-EDTA and then flooded with 2 ml trypsin-EDTA for 1 - 2 min each. After the cells detached, the flasks were shaken vigorously and then fresh L-15 plus 10 % FBS (L15-10) with the antibiotic-antimycotic mixture was added. When the cell monolayer became fully confluent, single flask monolayers were split with trypsin-EDTA as described earlier but subcultured onto more flasks at a cell density ratio of 1:2 - 1:3.

Effect of Temperature on Snakehead Spleen (SHS) Cell Cultures

SHS cells were seeded in 25-cm^2 tissue culture flasks and incubated at different temperatures of 5, 15, 25 and 40 °C for 1 week. In another experiment, 24 h confluent cultures of SHS cells grown in 25-cm^2 flasks at 25 °C were moved to different

temperatures of 15, 20, 25, 30 and 37 °C and incubated for 1 week. In both experiments, cell proliferation was monitored daily.

Cryopreservation

Confluent 1 day old cells were trypsinized with trypsin-EDTA, 4 ml L15-10 medium was added and the mixture centrifuged at low speed in a refrigerated centrifuge at 4 °C. The supernatant medium was discarded and the packed cells resuspended gently in a mixture of 90 % FBS and 10 % dimethylsulfoxide (DMSO). The cells were then subjected to gradual cooling at 4 °C for 1 h, then frozen to -10 °C for 1 h and finally stored at -70 °C (Plumb and Bowser 1983). For liquid nitrogen storage, the cells were mixed with minimum essential medium (MEM) containing 10 % FBS (MEM-10) with 10 % DMSO and immersed in liquid nitrogen.

Viable cells were recovered from frozen storage by holding the vials in running tap water at approximately 25 °C. Thawed cells were immediately transferred to a culture flask with L15-10 or MEM-10.

Susceptibility to the 91-97 Virus

Established cell cultures of SHS, snakehead liver (SHL) and catfish spleen (CFS) in 25-cm^2 flasks were inoculated with a suspension of 1.5 ml of a 10^6 TCID50 per ml virus that was isolated from an EUS fish in 1991 (#91-97). After 1 h, Leibovitz medium with 4 % FBS (L15-4) was added and then cells incubated at 25 °C for 1 week. The flasks were examined microscopically daily for cytopathic effects (CPE).

RESULTS

Of the techniques used, the direct inoculation of organ explants in culture flasks containing medium described as method B, consistently yielded successful primary cell cultures from the gonad, heart, kidney, liver and spleen of both catfish and snakeheads. Method A yielded poor explant outgrowths while methods C and D were successful for some but not all of the tissues (Table 5.1). All attempts to derive primary cell cultures from the brain, fins, skin and whole fry were unsuccessful. Initial outgrowths of cells from explants were detected 5 - 23 d after initial culture. Extensive cell migration was observed 9 - 28 d after initial inoculation.

The first passages of the primary cell cultures from catfish and snakeheads were conducted on d 29. The first cell culture passages, P-1 to P-2, took approximately 23 d to achieve at least 80 % confluency when incubated at 25 °C. Cell culture passages, P-2 to P-3, took 3 d for cells to develop complete confluency. On the fourth passage complete confluency within 24 h was observed. This rapid cell multiplication was consistently observed in subsequent passages.

Cell cultures were successfully established from the spleen, kidney, heart and gonad of catfish. Passage numbers of the cells are to date: catfish gonad (CFG) P-56, catfish heart (CFH) P-51, catfish kidney (CFK) P-7, catfish liver (CFL) P-8, catfish spleen (CFS) P-54 (Table 5.2). The CFL cells were lost to contamination at P-8 before these could be cryopreserved. Microscopically, catfish cells were initially a mixture of epithelioid and fibroblastic cells. After several passages, microscopic observations showed fibroblastic shaped cells rapidly propagating and becoming completely

Table 5.1. Primary cell culture development from different organs of catfish (*Clarias batrachus)* and snakeheads (*Ophicephalus striatus*) using four different processing methods, cultured in Leibovitz medium supplemented with 15 % fetal bovine serum and antibiotics and antimycotic incubated at 25 °C.

Fish Species	Organ	Method			
		A	B	C	D
Clarias batrachus	brain	-	-	-	-
	fins	-	-	-	-
	gonad	-	+	+	-
	heart	-	+	+	+
	kidney	-	+	-	-
	liver	-	+	-	+
	skin	-	-	-	-
	spleen	-	+	+	-
Ophicephalus striatus	brain	-	-	-	-
	fins	-	-	-	-
	gonad	-	+	-	+
	heart	-	+	+	+
	kidney	-	+	+	-
	liver	-	+	+	-
	skin	-	-	-	-
	spleen	-	+	+	+

A explants were placed onto the surface of tissue culture flask to attach for 1 h before adding L15-15 medium with antibiotic/antimycotic

B explants into tissue culture flask already containing L15-15 medium

C minced organ fragments pushed through astainless stell sieve using a syringe plunger into Petri dish with L15-15 medium

D minced tissue fragments exposed to 0.5% trypsin-EDTA with agitation for 1 h

\- no cell proliferation detected

\+ primary cell proliferation developed

confluent within 24 h of passage after P-3 at 25 °C. Cell density was estimated using a hemacytometer. Passage of a cell density of at least 2 x 10^2 cells per ml or one-third of a monolayer on a flask was required for complete confluency. Cell passage every 7 to 10 d was required to maintain viability of the cells. Consequently, cell culture from representative fish organ cell cultures was subcultured once a week.

Cell cultures from snakehead spleen, kidney, heart, gonad and liver tissues were successfully grown as monolayer cultures in-vitro. Passage numbers of the cells are to date: snakehead gonad (SHG) P-26, snakehead heart (SHH) P-22, snakehead kidney (SHK) P-19, snakehead liver (SHL) P-49, snakehead spleen (SHS) P-76 (Table 5.2). The snakehead cells were likewise initially a mixture of epithelioid and fibroblastic cells. After several passages these became consistently fibroblastic, multiplied rapidly and became completely confluent within 24 h of passage at 25 °C incubation. One third of flask cell density or 2 x 10^2 cells per ml, when passed in fresh L15-10 medium, achieved full confluency within 24 h of passage after P-3. Cell passage at 7 to 10 d was required to maintain viability of the cells. Subsequently, cell cultures were passed once each week.

Newly seeded SHS cells grew to complete confluency in 24 h at 25 °C but were killed at temperatures of 5 and 40 °C. At 15 °C, the cells grew slowly and reached only 50 % confluency after 3 - 5 d incubation. Confluent cells showed tolerance to temperatures of 15, 20, 25, 30 and 37 °C. However, at 15 °C, the cells started to peel off 24 h post-seeding with the monolayer completely peeling off at d 5. At 37 °C, monolayer detachment from the flask surface occurred on d 6. At 20 °C incubation, the cell passage interval could be extended to 2 weeks.

Table 5.2. Tissue cultures established at 25 °C from various organs of catfish (*Clarias batrachus*) and of snakeheads (*Ophicephalus striatus*).

	Organ Source	Passage Number
Clarias batrachus	gonad	56
	heart	51
	kidney	7
	liver	8
	spleen	54
Ophicephalus striatus	gonad	26
	heart	22
	kidney	19
	liver	49
	spleen	74

All established cells suspended in FBS with 10 % DMSO and stored at -70 °C remained viable for at least a year. Cells suspended in MEM-10 and stocked in liquid nitrogen have remained viable for the past 5 years.

The CFS, SHS and SHL cells were susceptible to the #91- 97 virus isolate. CPE developed 3 - 5 d after viral inoculation. Cells became granular, rounded and eventually detached from the flask surface and lyzed.

DISCUSSION

The report of snakehead cells established from the gonad, kidney, heart, liver and spleen is the first report of such cells established from the organs of this fish species. Frerichs *et al.* (1991) used snakehead cells derived from fry and caudal peduncle of snakeheads to isolate a rhabdovirus.

The CFS, SHS and SHL cells developed CPE within 3 d of exposure to isolate 91-97. The susceptibility of these established cells makes them excellent potential hosts for the isolation, propagation and *in vitro* study of viruses associated with EUS-affected fish.

The establishment of cell lines from the kidney, gonad and gills of catfish has been previously reported (Noga and Hartmann 1981). Cells established from the heart, liver, and spleen of catfish in this study are now reported for the first time.

All of the cell cultures established from catfish and snakeheads were readily trypsinized within 2 min, and multiplied rapidly when seeded in fresh L15-10 medium, becoming confluent in 24 h. Cells can also be adapted to medium with 4 % FBS. Although the cells can be cultivated in both L-15 and MEM, it was more practical to use

L-15 because of its phosphate buffer and ability to maintain a stable pH. Optimum temperature for SHS cell growth was at 20 - 25 °C.

Because of their fast growth rate, it became disadvantageous to maintain the cells in the laboratory as passage of the cells need to be performed every 2 weeks at 20 °C or every 7 - 10 d at 25 °C. In addition, the adherence of the cells on the surface of cell culture flasks was weak compared to that of other fish cell lines such as EPC (epithelioma papulosum cyprini) or CCO (channel catfish ovary) (unpublished data).

The cells established in this study were very sensitive to agitation or movement of the medium over the cell culture flasks, causing cells to detach and float in the medium. Hence, transport of these cells is problematic, as viability of the detached cells cannot be ensured. Moreover, cell attachment can be weakened after prolonged incubation at temperatures as low as 15 °C. Alternatively, cells frozen at -70 °C may be transported in dry ice or in liquid nitrogen.

The potential usefulness of the cell cultures established from catfish and snakeheads in this study is not limited to investigations on EUS-associated virus. Since fish viruses generally have a predilection for organs like the spleen, kidney, liver and gonad, availability of these cell cultures allows more choice of cell lines for screening fish epizootics of uncertain viral etiology. In addition, since research on warmwater viral infections of tropical fishes is increasingly important, these cell cultures from catfish and snakeheads may greatly facilitate initial investigations on viral etiology of infections in other fish species. Lastly, these cell cultures have potential application as bioindicators of aquatic pollutants in toxicology research (Martin-Alguacil *et al.* 1991).

CHAPTER 6

PRIMARY ISOLATION OF VIRUSES ASSOCIATED WITH EPIZOOTIC ULCERATIVE SYNDROME (EUS)-AFFECTED FISHES AND ENZOOTIC OBSERVATIONS ON THE DISEASE IN THE PHILIPPINES

INTRODUCTION

Frerichs *et al.* (1986) reported the isolation of a rhabdovirus from snakeheads (*O. striatus*) and freshwater eels (*F. alba*) from EUS outbreaks in Thailand and in Myanmar. About the same time, a birnavirus was isolated from sand goby, *Oxyeleotris marmoratus*, with EUS in Thailand by Hedrick *et al.* (1986). Subsequently, other workers reported the isolation of a rhabdovirus from snakeheads with EUS in Thailand (Kasornchandra *et al.* 1991). Primary isolation of viral pathogens from EUS-affected fishes was achieved using cell cultures established from EUS-susceptible snakeheads and catfish (*C. batrachus*). It was uncertain if these viruses were the primary cause of EUS, especially as Roberts (1993) mentioned that the viruses do not cause EUS.

In the Philippines, no isolations of virus from EUS-affected fishes have been attempted. The objective of this study was to isolate viruses associated with EUS-affected fishes and to assess the characteristics of the viruses in terms of prevalence by fish

species, organ source, province and season. Tests for the presence of virus neutralizing antibodies in fish were also conducted, as were tests to establish the daily incidence of EUS in Laguna de Bay during an epizootic.

MATERIALS AND METHODS

Sampling Sites/Fish Species

A total of three apparently healthy and 68 EUS-affected fish specimens were collected from two lakes (Laguna de Bay, Laguna and Naujan Lake, Mindoro), a river (Pampanga) and a freshwater earthen fishpond (Pangasinan) in the Philippines during the 1990-91 EUS outbreak. Fish samples consisted of snakeheads (100 - 500 g), climbing perch, *Anabas* sp. (40 - 90 g), gourami, *T. pectoralis* (25 - 45 g), catfish, *C. batrachus* (100 g) and *Tilapia* sp. (50 g).

In addition, two naive snakeheads and 12 catfish from Iloilo, an EUS-free area, were sampled. Fish samples were also collected in June and August 1991 when the disease was not apparent in enzootic areas. Such samples consisted of 22 snakeheads from Iloilo (non-enzootic area), three from Laguna de Bay, four from Mindoro and six from Pangasinan (enzootic sites) and eight catfish from Iloilo.

Data were also collected on the daily incidence of the disease in Laguna de Bay during January 1997. Eleven fishermen in Laguna de Bay monitored the number of EUS fish among their daily catch of snakeheads in Laguna de Bay using bamboo/wire fish traps.

EUS Lesion Grading

EUS fish samples were classified by lesion type according to the lesion grade criteria set in Table 2.2.

Tissue Sampling for Virus Detection

Each fish was lethally anaesthesized using tricaine methane sulfonate (MS222), and surface sterilized with a cotton swab soaked in 70 % ethanol before dissection. Tissues were aseptically sampled from external lesions and from visceral organs (pool of spleen, liver, kidney and gills). Tissue samples from one to two fish were pooled and transported in Leibovitz medium plus 200 I.U. penicillin per ml and 200 ug streptomycin per ml of medium in sterile culture tubes kept on ice. These were stored at -70 °C until processed.

Each tissue sample was aseptically and completely drained of its transport medium, weighed, then diluted to 1:5 – 1:10 (Chong *et al.* 1990) with Eagle's balanced salt solution (EBSS) containing an antibiotic/antimycotic mixture (200 I.U. penicillin G sodium, 200 ug streptomycin sulfate, 50 ug amphotericin B per ml medium) and homogenized with mortar and pestle. Homogenates were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant of each sample was then filtered through a 0.45 um pore size membrane filter (Millipore) and stored at -70 °C until assayed for virus.

Tissue Culture and Viral Isolation

Snakehead spleen cells (SHS) were grown in Leibovitz medium supplemented with 10 % fetal bovine serum (FBS) (L15-10) in tissue culture flasks (Falcon) at 25 °C.

For primary viral isolation, the cells were seeded into 24-well plates (Falcon) with Leibovitz medium plus 4 % FBS (L15-4). After 24 h incubation when cells were fully confluent, the media was aseptically removed and 100 ul of each filtrate was gently inoculated onto the cell monolayer. The plates were rocked gently for a few seconds and then incubated for 1 h at 22 - 25 °C after which each well was overlaid with 1.0 ml L15-4 buffered with 1M N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid (HEPES). The plates were then incubated at 25 °C for 7 d. The plates were examined microscopically daily for cytopathic effects (CPE). Blind passages were carried out on negative samples at least three times. Only those isolates manifesting consistent 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. All media were supplemented with antibiotic-antimycotic (100 I.U. penicillin G sodium, 100 ug streptomycin sulfate, 25 ug amphotericin B per ml of medium).

Collection of Fish Sera

Blood of apparently healthy and EUS-affected snakeheads from Laguna de Bay at the peak of the EUS season in 1995-96 as well as from naive snakeheads in Iloilo were collected by cardiac puncture using sterile syringes. Blood samples were allowed to clot in sterile tubes overnight at 4 °C. Serum was separated after low speed centrifugation at 1500 rpm for 15 min. All serum samples were frozen at -70 °C until neutralization tests could be conducted.

Neutralization Test

Serum samples were thawed quickly and then serially diluted two-fold with Hank's balanced salt solution (HBSS) containing 200 I.U. penicillin G sodium, 200 ug streptomycin sulfate, and 50 ug amphotericin B per ml. An equal volume of a 10^5 $TCID_{50}$ per ml of the 95-96 virus isolate was added to each dilution and incubated for 1 h at 25 °C. Thereafter, 10 ul of each serum-virus mixture was added to monolayers of CCO cells (kindly provided by J. Plumb, Auburn University) in 96-well tissue culture plates (Falcon) in quadruplate wells. After 1 h incubation at 25 °C, 100 ul of minimum essential medium (MEM) supplemented with 4 % FBS (MEM-4) and HEPES were gently added to each well. The plates were then incubated at 25 °C for 5 d and examined microscopically for CPE. Neutralization titer was estimated based on the method of Rovozzo and Burke (1973).

Statistical Tests

Chi-square analyses applying Fisher's Exact Test (two-tail) was used in analyzing the data on virus isolation by lesion grade, fish species, lesion/visceral sources, and province. Significant differences of the proportions were evaluated by the Student's *t*-test. The data on neutralizing antibodies were analyzed using the analysis of variance (ANOVA).

RESULTS

Approximately 35 % of the total number of sampled EUS-affected fishes were positive for virus (Table 6.1). Among apparently healthy (AH) fishes, 67 % were positive for virus. About 44 % and 75 % of grades I and II lesioned fishes, respectively, were virus positive. Among samples with grades III and IV lesions, only 23 % and 14 % of the cases, respectively, were virus positive. Of the total sampled fishes including apparently healthy fish, those with grade II lesions yielded the highest frequency of positive virus isolation.

Comparing the positive and negative virus isolation rates among various stages of EUS lesioned fish samples, Chi-square tests showed that the presence/absence of virus was highly significantly ($p<0.0005$) related to lesion grade. Significant differences were noted between grades II vs III and IV ($p<0.001$) as well as among apparently healthy and grades I vs IV ($p<0.05$). All fish samples from Iloilo, a non-enzootic site, were negative for virus.

Except for *Tilapia* sp., all other fish species tested positive for virus (Table 6.2). *Anabas* sp., however, yielded the highest virus isolation frequency rate (14 %) among the total number of fishes sampled. Differences in virus frequency rate among fish species were not statistically significant ($p>0.05$).

There was no significant difference ($p>0.05$) between muscle lesion and visceral samples in the occurrence of virus (Table 6.3). In addition, snakehead samples from all provinces, except Iloilo, were positive for virus (Table 6.4).

Prevalence of the virus when the disease has not previously been reported is tabulated in Table 6.5. The results showed that only one of six fish in Pangasinan and none in all other provinces were positive for virus at those times.

Table 6.1. Primary virus isolations from apparently healthy (AH), and epizootic ulcerative syndrome (EUS)-affected snakeheads *(Ophicephalus striatus), Anabas sp., Trichogaster sp.* and Tilapia sp. by lesion grade from an EUS enzootic site, Pangasinan, during the 1990-91 EUS outbreak.

Lesion Grade*	Virus	
	Positive	Negative
AH	2	1
I	4	5
II	12	4
III	5	17
IV	3	19
Total	25	46

* see Table 2.2 for lesion grade criteria

Table 6.2. Primary virus isolations from muscle lesion and viscera of epizootic ulcerative syndrome (EUS)-affected *Ophicephalus striatus, Anabas sp.* and *Trichogaster sp.* collected from an EUS enzootic site, Pangasinan, by fish species during the 1990-91 EUS outbreak.

Fish Species	Virus	
	Positive	Negative
Ophicephalus striatus	4	16
Anabas sp.	8	14
Trichogaster sp.	5	9
Tilapia sp.	0	1

Table 6.3. Primary virus isolations from muscle lesions and viscera of epizootic ulcerative syndrome (EUS)-affected *Ophicephalus striatus*, *Anabas* sp. and *Trichogaster* sp. collected from EUS endemic sites of Laguna de Bay, Mindoro, Pampanga and Pangasinan during the 1990-91 EUS outbreak.

Fish Species	Virus Positive		Virus Negative	
	Lesion	Viscera	Lesion	Viscera
Ophicephalus striatus	2	5	4	1
Anabas sp.	8	6	0	2
Trichogaster sp.	3	2	2	3
Subtotal	13	13	6	6

Table 6.4. Prevalence of virus among naive catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus)* from a non-endemic site, Iloilo and among epizootic ulcerative syndrome (EUS)-affected fishes including apparently healthy snakeheads from EUS endemic provinces of Laguna de Bay, Mindoro, Pampanga and Pangasinan during the 1990-91 EUS outbreak.

Province	Virus	
	Positive	Negative
Iloilo	0	14
Laguna de Bay	3	0
Mindoro	2	3
Pampanga	3	3
Pangasinan	17	57
Total	25	77

Table 6.5. Prevalence of virus among naive catfish (*Clarias batrachus*) and snakeheads (*Ophicephalus striatus*) in May - August 1991 when epizootic ulcerative syndrome (EUS) was not apparent on the fish in Laguna de Bay, Mindoro and Pangasinan.

Province	Virus	
	Positive	Negative
Iloilo (Naive)	0	22
Laguna de Bay	0	3
Mindoro	0	4
Pangasinan	1	5
Total	1	34

Apparently healthy and grade I lesioned snakeheads had the highest number of fish (5 of 15; 5 of 20, respectively) showing the presence of detectable neutralizing antibodies (Table 6.6). Also, the largest mean titer of neutralizing antibodies was detected among apparently healthy and grade I lesioned groups of snakeheads. Grades II and I fish had neutralizing antibody prevalence of 25 and 29 %, respectively. All 20 serum samples from grade IV lesioned snakeheads showed no neutralizing antibodies but 10 % of naive and grade III fish had neutralizing antibodies. However, no significant differences ($p>0.01$) in neutralizing antibody titer were observed between lesion grades. Overall, most fish serum samples were negative for detectable neutralizing antibodies.

Prevalence of EUS-affected individuals among total snakeheads caught by the fishers in Laguna de Bay during the EUS season of 3 to 31 January 1997 ranged from 0 to 100 % (Table 6.7). However, only 10 % and 2 % of the frequency distribution of catch data had 0 and 100 % EUS prevalence, respectively. The mode of EUS catch fell at the frequency distribution range of 11 - 15 % while 50 % of catch data had 15 - 25 % prevalence with an estimated mean of 18 %. Only 5 % of EUS catch data had more than 40 % prevalence. The general mean prevalence was 20.16 through the January 1997 samplings. There were no significant differences ($p>0.01$) among day-to-day EUS prevalence.

DISCUSSION

Frerichs (1994) reported a 5 and 0 % isolation rate of virus from EUS fish sampled in Thailand and Bangladesh, respectively. Such low and inconsistent rates of

Table 6.6. Neutralizing antibody titers in (1) naive, (2) apparently healthy (AH) and (3) epizootic ulcerative syndrome (EUS)-affected snakeheads (*Ophicephalus striatus*) from Laguna de Bay during the 1995 - 96 EUS outbreak.

Fish No.	NA Titer of EUS-affected Fish with Lesion Grades					
	Naive	AH	I*	II	III	IV
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	2.75	0
4	0	2	0	6.71	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	4.7	0	0	0	0	0
8	0	10.47	0	0	0	0
9	0	9.19	0	0	0	0
10	0	4.54	6.34	4	0	0
11	0	0	9.19			0
12	0	4.07	6.98			0
13		0	0			0
14		0	10.67			0
15		0	0			0
16			0			0
17			0			0
18			6.35			0
19			0			0
20			0			0
Mean	0.39	2.02	1.98	1.07	0.28	0
Prevalence	8%	33%	25%	20%	10%	0%

NA neutralizing antibody
* see Table 2.2 for lesion grade criteria

Table 6.7. Percent snakeheads (*Ophicephalus striatus*) with epizootic ulcerative syndrome (EUS) lesions trapped by 11 fishermen in Laguna de Bay during the EUS season in January 1997.

January	Name of Fisherman											Daily
1997	Fred	Arik	Bian	Sipek	Benny	Popoy	Sixto	Eddie	Pedring	Siding	Jaime	% Mean
3	33	12	32	no data	38	33	27	no data	39	27	60	46.50
4	17	19	11	77	12	34	38	no data	no data	20	24	20.50
5	15	0	23	17	0	31	27	31	no data	14	30	22.50
6	13	0	28	53	24	34	18	23	no data	36	22	17.50
7	24	22	9	13	28	18	22	29	23	91	19	21.50
8	26	9	26	22	30	19	29	36	21	60	20	23.00
9	16	16	28	0	11	no data	23	21	no data	0	no data	16.00
10	14	15	25	100	38	8	33	21	14	30	no data	14.00
11	9	13	24	no data	0	no data	20	no data	no data	22	23	16.00
12	no data	no data	no data	no data	0	22	no data	17	10	36	no data	17.00
13	no data	no data	11	44	12	no data	no data	no data	no data	31	19	23.40
14	0	17	0	15	11	22	28	0	21	0	13	11.55
15	5	7	15	0	0	no data	no data	0	no data	100	0	15.88
16	22	4	0	14	22	21	no data	25	13	15	24	16.00
17	34	16	13	no data	33	no data	38	8	no data	10	12	20.50
18	18	7	27	no data	14	25	32	16	9	no data	11	17.67
19	no data	no data	no data	22	no data	no data	no data	16	no data	21	no data	19.67
20	8	0	19	86	6	55	0	13	12	no data	11	21.00
21	15	6	30	100	3	no data	no data	no data	no data	0	no data	25.67
21	19	0	35	33	4	12	0	26	9	no data	5	14.30
23	21	0	no data	0	14	no data	20	no data	no data	14	no data	11.50
24	29	17	26	31	8	27	39	no data	18	no data	14	23.22
25	17	8	26	13	10	no data	no data	no data	no data	18		15.33
26	no data	no data	no data	no data	no data	30	30	14	21	no data	17	22.40
27	14	12	19	4	15	no data	25	no data	no data	40	no data	18.43
28	12	10	15	10	17	22	17	18	17	no data	5	14.30
29	13	13	20	6	13	no data	22	no data	no data	32	no data	17.00
30	8	14	14	5	8	26	25	31	no data	no data	18	16.56
31	8	8	no data	no data	no data	no data	no data	no data	no data	no data	no data	8.00
% Mean	16.4	18.11	19.83	30.23	14.27	25.82	24.43	19.68	17.07	29.38	18.26	20.15

virus recovery from fish showing signs of EUS tend to preclude acceptance of a virus being the causative agent of EUS (Frerichs 1994).

In this study, virus was isolated from 35 % of 71 fishes showing signs of EUS. This is significant since to achieve a 95 % probability of detecting at least one reactor from a population with an apparent prevalence of 5 %, the total sample size required should be in excess of 60 fish (Thorburn 1996).

Virus isolation by cell culture techniques is more sensitive than by immunological assays (Sanz and Coll 1992). The higher success rate of virus recovery in this study in comparison to Frerichs *et al.*'s (1989a) results, is likely due to the low dilution (1:5 - 1:10) of the tissue filtrates used. Tissue toxicity was ruled out on subsequent passages. Frerichs *et al.* (1989a) in isolating the viruses from EUS fish in Thailand diluted the tissue filtrates 100-fold. According to Sanz and Coll (1992), virus can be detected only if it is present in excess of 10^3 $TCID_{50}$ per ml.

Sixty-seven percent of apparently healthy fishes from EUS enzootic areas can harbor the virus and perhaps serve as carriers of the EUS-associated virus. Other laboratories monitoring asymptomatic salmonid populations for surveillance and certification purposes have shown an apparent prevalence of infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV) as often less than 5 % (Thorburn 1996). Similarly, pike fry virus has been isolated from pike, *Pseudorasbora parva* (Ahne and Thomsen 1986) and birnavirus from turbot, *Scophthalmus maximus* (Novoa *et al.* 1995) neither of which showed any signs of disease. Since Frerichs *et al.* (1989b) did not recover virus from any apparently healthy fish

removed from areas enzootic for EUS, this may possibly be attributed to the high dilution of the tissue filtrates that they used.

Fish with EUS lesions of grades II and I yielded a significantly ($p<0.0005$) greater success rate in virus isolation compared to more severely lesioned EUS fishes. In fact, the success rate of positive virus isolations was highest among EUS-affected fishes with grade II lesions. These observations sustain the hypothesis that intensification of lesion development, such as that observed in grades III and IV EUS lesioned fish, is probably a result of a superimposed secondary bacterial infection due to *A. hydrophila* (Chapters 2, 3 and 4) or due to fungal infection with *Aphanomyces* sp. (Roberts *et al.* 1993).

Mulcahy *et al.* (1983b) and Plumb *et al.* (1981) have reported difficulties in isolating virus in the absence of acute signs of disease. The development and persistence of grades III and IV lesions among EUS-affected snakeheads likely represent the chronic phase of the disease. This may partly explain the low success rate of virus isolations. In addition, Tate *et al.* (1990) have reported that IPNV infection causes suppression of both "T"-like and "B"-like leukocyte activities in rainbow trout (*O. mykiss*). Such impairment of the immune system may cause a depletion of NA levels to undetectable titers as observed in grade IV lesioned fish. Finally, Kamei *et al.* (1988) detected the presence of anti-IHNV activity in 190 strains of bacterial isolates which induced >50 % plaque reduction. These bacteria consisted of *Pseudomonas, Aeromonas, Vibrio* and *Coryneforms*. *Aeromonas* and *Pseudomonas* which were among the predominant bacteria associated with EUS lesions may likely secrete anti-viral substances against the EUS associated virus, which may partly explain the low detection rate of virus in severely lesioned EUS fish.

The virus was isolated in all fish species sampled except *Tilapia* sp. and *C. macrocephalus* (Table 6.3). This confirms the wide host range of the EUS-associated virus deduced from EUS signs among a variety of fish species in the Philippines (Palisoc 1990) and elsewhere (Roberts *et al.* 1986).

Among the fish species found positive for the virus, *Anabas* sp. appeared to yield the highest success rate for virus isolation. Perhaps, *Anabas* sp. is a more susceptible host than snakeheads and gourami. However, snakeheads and catfish remain the most economically important species affected by EUS. That *Tilapia* sp. was negative correlates with reports that tilapia are refractory to EUS (Ahmed and Rab 1995; Mines and Baluyot 1986; Roberts *et al.* 1986). However, only a few tilapias were examined in the present study.

The virus was isolated from muscle lesions and viscera of lesioned fishes at very similar rates. Thus, future attempts to isolate the EUS-associated virus from EUS-affected fishes need not use both tissue samples, thereby reducing sampling cost and effort.

Viruses were isolated from snakeheads collected from sampling sites of Laguna de Bay, Laguna; Naujan Lake, Mindoro; freshwater ponds/rivers in Pampanga and Pangasinan which were all known to be EUS enzootic provinces. This confirms the enzooticity of the virus in these areas. No virus was isolated from snakehead and catfish samples in Iloilo confirming this province as an EUS-free area.

These results suggest that aquatic pollution is not a contributing factor to EUS development as the waters of Naujan Lake and the Pangasinan or Pampanga sites are unpolluted yet contain EUS lesioned fishes (Bondad-Reantazo *et al.* 1994; Phillips 1994).

Only 1 of 13 sampled snakeheads from enzootic areas, during the period when the disease was not apparent, was positive for virus. This indicated that the virus might persist among snakeheads in enzootic areas. Persistence may be in low titers; hence the success rate of virus isolation may be quite low. Earlier, Mulcahy *et al.* (1983b) and Plumb *et al.* (1981) reported difficulty in isolating virus in the absence of the acute phase of the disease. The sampled snakeheads may have been survivors of the most recent EUS outbreak and may well have been carriers of the virus. The carrier state may extend for long periods following exposure to viral pathogens. This is true, for example for IPNV where release of IPNV fluctuates with time (Hedrick and Fryer 1982). The present observations suggest a latent infection in fish that survive infections as juveniles. Such carrier fishes likely play an important role in the interspecies and interseasonal spread of the virus.

The presence of neutralizing antibodies (NA) indicates that the EUS-associated virus elicits an immune response although the titers are low. Yamamoto (1975) likewise, obtained low NA levels among brook trout (*Salvelinus fontinalis*) infected with IPNV. An interesting observation was the lack of NA in all Grade IV lesioned snakeheads and the higher frequency of fish samples with NA among apparently healthy and grade I lesioned fish. It is possible that severely lesioned EUS snakeheads represent fish that have a poor antibody response to EUS virus. In general, the mean titers of the NA showed a decreasing trend from apparently healthy to Grade IV lesioned fish. This may imply that such fish are protected against the virus by having an effective antibody response. Whether these NA actually have protective effects is, however, not known. Traxler *et al.* (1997) determined that when IHN virus neutralizing activity was detected in serum, the

prevalence of detectable virus was significantly less ($p<0.001$) than when anti IHN-virus antibodies were absent. Anti-CCV NA in the sera of channel catfish were also low even from those found positive for CCV (Hedrick and McDowell 1987). On the other hand, Hattenberger-Baudouy *et al.* (1989) reported that anti-IHNV NA can persist for up to 48 weeks in rainbow trout (*Oncorhynchus mykiss*). If similar persistence occurs in snakeheads, it is possible that NA titers from the previous EUS outbreak (up to February) were already declining at the time serum samples were taken in January of the following year. This may explain the low NA titers obtained. Also, declining water temperature prevalent during the EUS season may have influenced the intensity of the antibody response in the snakeheads.

Immunological suppression after viral infection occurs in fish. Rainbow trout were reported to suffer immunosuppression after infection with IPNV (Tate *et al.* 1990). Furthermore, the fact that EUS occurs only during the coldest months of the year strengthens the immunosuppression theory. Bowser and Munson (1986) reported that temperature affected the anti-CCV NA response in channel catfish: titers declined in winter and rose again in spring or summer. A similar scenario was hypothesized for EUS since the disease occurs only during the cool months of the year when the immune response of affected fishes is likely compromised (Roberts *et al.* 1986). The presence of viral NA may indicate the presence of a previous exposure to the EUS-associated virus as has been reported among channel catfish previously exposed to CCV (Plumb 1973).

It is also possible that the very low neutralizing antibody detection success in this study may have been due to the short 1 h incubation period. Hattenberger-Baudouy *et al.* (1989) reported that increasing the incubation period for the serum-virus interaction from

1 h to 16 h increased the sensitivity of the test. Without complement, detection of viral neutralization is possible if NA titers are high enough to provide protection or antibody detection (Hedrick *et al.* 1992).

Outbreaks of EUS have been recurring annually in several areas of the Philippines since first reported in Laguna de Bay in 1985-86 (Mines and Baluyot 1986). Documented reports show that the prevalence of EUS among snakeheads varied from 1 to 59 % in January and February 1986 in Laguna de Bay (Natividad 1986; Mines and Baluyot 1986). In the 1988-89 EUS epizootic, a 76.4 % infection rate among snakeheads was reported (Palisoc 1990). In the 1995-96 EUS season, a 1 d sampling of snakehead showed an approximate 50 % prevalence (Laguna de Bay fishermen, pers. comm.). In that same year, harvest data from a freshwater pond showed that the prevalence among snakeheads was 39 % (J. Paclibare, Bureau of Fisheries and Aquatic Resources, Philippines, pers. comm.).

In the present study, prevalence of EUS among snakeheads in January 1997 had a wide range of 0 - 100 % with an overall mean of 20.2 %. The daily mean prevalence varied from 8 - 46.5 % with its mode at 11 - 25 % and median at 23 %. The wide range in daily prevalence of EUS explains why fish farmers claim that the annual prevalence had not diminished (Laguna de Bay fishermen and Buencamino, pers. comm.). The data summary is the only report of a 1 month EUS record of daily prevalence during an EUS season (Table 6.7).

The foregoing data illustrate the continuing havoc that EUS is causing in the freshwater finfish fisheries and aquaculture industry in the Philippines. The more consistent and higher prevalence of virus in both wild and cultured EUS-susceptible

fishes than has been reported earlier suggests that virus is the primary pathogen in EUS. The importance of this in the EUS cannot be ignored.

CHAPTER 7

CHARACTERIZATION OF A VIRUS ASSOCIATED WITH THE EPIZOOTIC ULCERATIVE SYNDROME (EUS) IN SNAKEHEADS (*OPHICEPHALUS STRIATUS*) IN THE PHILIPPINES

INTRODUCTION

Bacteria and fungi have been found associated with pathogenicity in snakeheads (*O. striatus*) but they have not been shown conclusively to be the primary disease agents of EUS (Chapters 2, 3 and 4; Callinan at al. 1995a; Roberts *et al.* 1993). Rhabdoviruses and a birnavirus associated with EUS-lesioned fish were isolated in Thailand (Frerichs *et al.* 1986, Hedrick *et al.* 1986, Kasornchandra *et al.* 1991) but their virulence has not been established (Frerichs *et al.* 1993).

This study report on the characteristics of one of these virus isolates, #91-97, from an EUS-affected snakehead collected from Laguna de Bay, Philippines, in January 1991.

MATERIALS AND METHODS

Viruses

The test virus, #91-97, was isolated from several virus-positive filtrates obtained from severely lesioned EUS-affected snakeheads. Fish samples had mean weights of 175 g and were collected from Laguna de Bay, Philippines, in January 1991 as described in Chapter 6. J. Fryer (Oregon State University) provided the snakehead rhabdovirus (SHRV), while the infectious hematopoietic necrosis virus (IHNV) came from G. Traxler (Pacific Biological Station, Nanaimo).

Cell Cultures

Several cell line cultures were used in these experiments namely SHS, CFS, Atlantic salmon (AS), bluegill fry (BF2) (Wolf and Quimby 1966), channel catfish ovary (CCO) (Bowser and Plumb 1980), chinook salmon embryo (CHSE), Epithelioma papulosum cyprini (EPC) (Fijan *et al.* 1983), and rainbow trout gonad (RTG-2). SHS and CFS established earlier (Chapter 5) were maintained in L15-10 in tissue culture flasks (Falcon) at 25 °C.

R. Hedrick (University of California, Davis) provided the CCO cells while G. Traxler provided the other cells. These cells were maintained in Minimum Essential Medium (MEM) with 10 % FBS (MEM-10). Incubation temperatures were at 25 °C for BF2, CFS, CCO, EPC, and SHS and 20 °C for AS, CHSE, and RTG-2. GIBCO media (L15 and MEM), reagents and antibiotics were used. The pH of the media was adjusted to 7.2 - 7.4 using 7.5 % sodium bicarbonate. All media were supplemented with an

antibiotic-antimycotic mixture (100 I.U. penicillin G sodium, 100 ug streptomycin sulfate, 25 ug amphotericin B per ml medium).

Virus Propagation and Assay

The 91-97 viral isolate and the SHRV were propagated in SHS cells in tissue culture flasks (Falcon) at 25 °C for 2 - 5 d while IHNV was grown in CHSE cells at 15 °C for 2 weeks. Each virus was adsorbed on confluent monolayers of cells for 1 h before addition of either L15-4 or MEM-4. When at least 90 % of cells had lysed, the medium was clarified by low speed centrifugation (2500 rpm) at 4 °C for 15 min and the supernatant stored in aliquots at -70 °C until used.

For viral assays, confluent cells seeded on 96-well plates (Falcon) were used. Ten-fold serial dilutions of the test virus were inoculated on confluent monolayers of cells (8 wells per dilution) for 1 h before addition of either L15-4-HEPES or MEM-4- HEPES (L15-4 or MEM-4 buffered with 1M N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid). The plates were then incubated appropriately and examined microscopically for cytopathic effects (CPE) (after 7 or 14 d in IHN inoculated CHSE cells). Viral titer was estimated by the end-point dilution assay ($TCID_{50}$) of Rovozzo and Burke (1973).

Susceptibility of Cell Lines

The susceptibility of different cell lines to the 91-97 virus isolate was tested using BF2, CFS, CCO, EPC, and SHS at 25 °C for 5 d and AS, CHSE, RTG-2 at 20 °C for 7 d. Viral titers were estimated for cell cultures manifesting CPE. Blind passages were conducted with SHS cells from the supernatants of the cell lines not showing any CPE.

Virus Replication at Different Incubation Temperatures

Confluent SHS cells in 25-cm^2 tissue culture flasks (Falcon) were inoculated with 1.5 ml a 10^3 $TCID_{50}$ per ml suspension of the 91-97 virus. The virus was adsorbed onto SHS cells at 25 °C for 1 h, washed and overlaid with L15-4. Seven inoculated flasks were incubated at each of the following temperatures: 15, 20, 25, 30 and 37 °C. Each day for 7 d, one 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 centrifugation, and the virus titer of each was determined using 96-well plates.

Stability of the Virus at Varying Temperatures

Clarified viral suspensions of the 91-97 virus were dispensed in 10 ml aliquots in sterile glass tubes and stored at various temperatures (°C): -10, 4, 20 and 30. After 3, 6, 10, 30, 60 and 120 d, the virus titers were assayed in 96-well plates.

Plaque Assay

Plaque assay of the 91-97 virus was conducted using 8 well plates (Nunc) with SHS cells following the method of Burke and Mulcahy (1980). At termination, the monolayers were overlaid with a formalin fixative-crystal violet stain solution, rinsed with water, drain-dried and the plaques counted. Virus concentrations were calculated and expressed as plaque forming units (pfu) per ml.

Growth Curve Determination

The growth curve of the 91-97 virus was determined on SHS cells grown in 25-cm^2 flasks. Confluent cells were inoculated with 1.5 ml a 10^3 $TCID_{50}$ per ml suspension of the 91-97 virus and incubated at 25 °C. At 2 h intervals for 24 h, four flasks were removed and the titer of cell free and total virus assayed. Cell-free virus titer was determined from the supernatant pool of two flasks following low centrifugation. Total virus was determined from a pool of two 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.

Effect of Chemical Agents, Heat and Repeated Thawing and Refreezing

The sensitivity of the virus to chloroform was determined according to the method of Feldman and Wang (1961). The viral suspension was treated with either chloroform or L15 medium for 10 min at 25 °C, centrifuged and the aqueous supernatant assayed for the virus. The test virus was likewise treated with 5-iododeoxyuridine (IUDR) or EBSS for its resistance to IUDR following Rovozzo and Burke's method (1973).

The effect of low pH on the virus was determined in L15 adjusted to pH 3 and pH 7 for 30 min exposure. The pH 3 treatment was neutralized with 0.1N NaOH before viral titration.

Virus titer was determined after exposure of the test virus to heat at 56 °C for 0.5, 1, 2, 4, 6 and 8 h.

The virus titer after each repeated freezing and thawing cycle was determined for up to three cycles.

All assays were conducted using 96-well plates with SHS cells. Virus titers were estimated according to the method of Rovozzo and Burke (1973).

Purification of the Virus

The 91-97 virus was grown in SHS cells supplemented with 2 % bovine serum albumin (BSA) (Sigma) following the method of Wunner (1985) until there was complete destruction of the monolayer. The suspending medium was then clarified by low speed centrifugation at 4 °C and the virus in the supernatant further purified by sucrose gradient ultracentrifugation according to the method of Engelking and Leong (1989). The virus band was washed and packed by ultracentrifugation and the purified virus was resuspended in Tris buffer before being stored at -70 °C.

Polyclonal Antibody Production

Antiserum against the purified 91-97 virus was produced by injection of New Zealand white female rabbits with the purified virus mixed with incomplete Freund's adjuvant. Approximately 100 ug of purified viral protein was injected subcutaneously into the rabbit. The rabbit was allowed to rest for 2 weeks after which it was boosted with 50 - 90 ug of the antigen. Blood was collected from the marginal ear vein, allowed to clot at 37 °C for 1 h and then incubated overnight at 4 °C. The serum was collected by centrifugation at 1500 rpm for 15 min at 4 °C then stored in aliquots at -70 °C. Normal rabbit serum was also collected before immunization as negative control.

Neutralization Test

Serological comparison of the 91-97 virus with other EUS-associated viruses i.e. SHRV and UDRV as well as the IHNV were conducted by neutralization tests using polyclonal antisera raised against the 91-97 virus isolate and UDRV (from Dr. N. Frerichs, Stirling University, UK).

Serum samples were thawed quickly and then serial two-fold dilutions were prepared with Hanks balance salt solution (HBSS) containing 200 I.U. penicillin G sodium, 200 ug streptomycin sulfate, 50 ug amphotericin B per ml. An equal volume of a 10^4 $TCID_{50}$ per ml of the 91-97 virus was added to each dilution and incubated for 1 h at 25 °C. Thereafter, 10 ul of each serum-virus mixture was added to monolayers of SHS cells in 96-well tissue culture plate wells (Falcon) in quadruplicate. After 1 h incubation at 25 °C, 100 ul of L15-4-Hepes was gently added to each well then incubated at 25 °C for 5 d. EPC cells were used for IHNV with MEM-4-Hepes incubated at 15 °C for 2 weeks. At termination, the plates were examined microscopically for CPE. Neutralization titers were estimated based on the method of Rovozzo and Burke (1973).

Electron Microscopy

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

Experimental Fishes

Healthy/naive snakeheads, 40 - 80 g, from Iloilo, Philippines were acclimated at ambient temperature (27 - 32 °C) for 1 week after transport from a river source. At least 3 d prior to the infection experiments, the naive fish were moved to a laboratory where the temperature was maintained at 20 - 25 °C. Fish were stocked in 5 liter aquaria containing cooled UV-sterilized unchlorinated freshwater. Two fish were stocked per aquarium.

Infection Experiments

Healthy snakeheads were exposed to the virus by bathing dermally-abraided test fish or unabraided fish, or by injection using intraperitoneal (IP), intrakidney (IK), intraspleen (IS) and intradermal (ID) routes of administration.

In another experiment, snakeheads and catfish were individually placed in 5 liter aquaria and inoculated with the 91-97 isolate intraperitonally, or by the bathing of skin-abraided fish.

In a separate experiment, snakeheads fed and unfed for 2 weeks prior to the experiment were inoculated with the 91-97 isolate. Test virus was also tissue-passaged in healthy snakeheads 1 to 3 times before challenge injection of the naive snakeheads. Lesion development and mortalities were monitored daily.

Reisolation of the Test Virus

Lesion/muscle and visceral organs were sampled, diluted to 1:10 and homogenized aseptically in Hanks balanced salt solution (HBSS) containing an antibiotic-antimycotic mixture (GIBCO). Homogenates were clarified by low speed

centrifugation in a refrigerated centrifuge at 4 °C then filtered through a 0.45 u membrane filter (Millipore). The filtrates were stored at -70 °C until viral assay.

RESULTS

Tests on different cell lines showed that BF2, CFS, CCO and SHS were susceptible to the 91-97 virus isolate, producing similar virus titers of 10^6 $TCID_{50}$ per ml (Fig. 7.1). In SHS cells, the virus induced CPE yielding 3.02 x 10^6 $TCID_{50}$ per ml at 25 °C in 2 - 3 d. The cytopathic effects on both CFS and SHS cells consisted of granulation, rounding up, followed by detachment and lysis of cells (Fig. 7.2). CPE was initially observed 24 h after viral inoculation, which caused complete destruction of the monolayer 2 - 3 d post viral exposure. In CHSE, the virus titer was approximately 1.35 x 10^3 $TCID_{50}$ per ml, but when blind passaged in SHS, a titer of 10^7 $TCID_{50}$ per ml was attained. EPC, AS and RTG-2 cells were refractory to the test virus. However, upon three successive blind passages in SHS, the virus yielded titers of 10^5, 10^3 and 10^4 $TCID_{50}$ per ml. Virus titer by plaque assay was $1.63x10^6$ pfu per ml.

Fig. 7.3 shows the 91-97 virus replication at various incubation temperatures. Optimum virus replication yielding titers of at least 10^6 $TCID_{50}$ per ml were achieved at 25 and 30 °C in d 1 and at 15 and 20 °C in d 2 with continuous high titers until d 6. At 15 °C, viral replication was initially slower but virus titers as high as 2.13 x 10^7 $TCID_{50}$ per ml occurred at d 4 to 7. There was no significant increase in virus titer at 37 °C, a titer of $TCID_{50}$ of 10^2 being maintained at d 1 to 5. Thereafter, the cells detached and the virus was inactivated by d 6.

Fig. 7.1. Titers of the 91-97 virus isolate after inoculation in bluegill fry cells (BF2), channel catfish ovary cells (CCO), catfish spleen cells (CFS) and snakehead spleen cells (SHS) incubated at 25 °C for 5 d and in chinook salmon embryo cells (CHSE) incubated at 20 °C for 7 d.

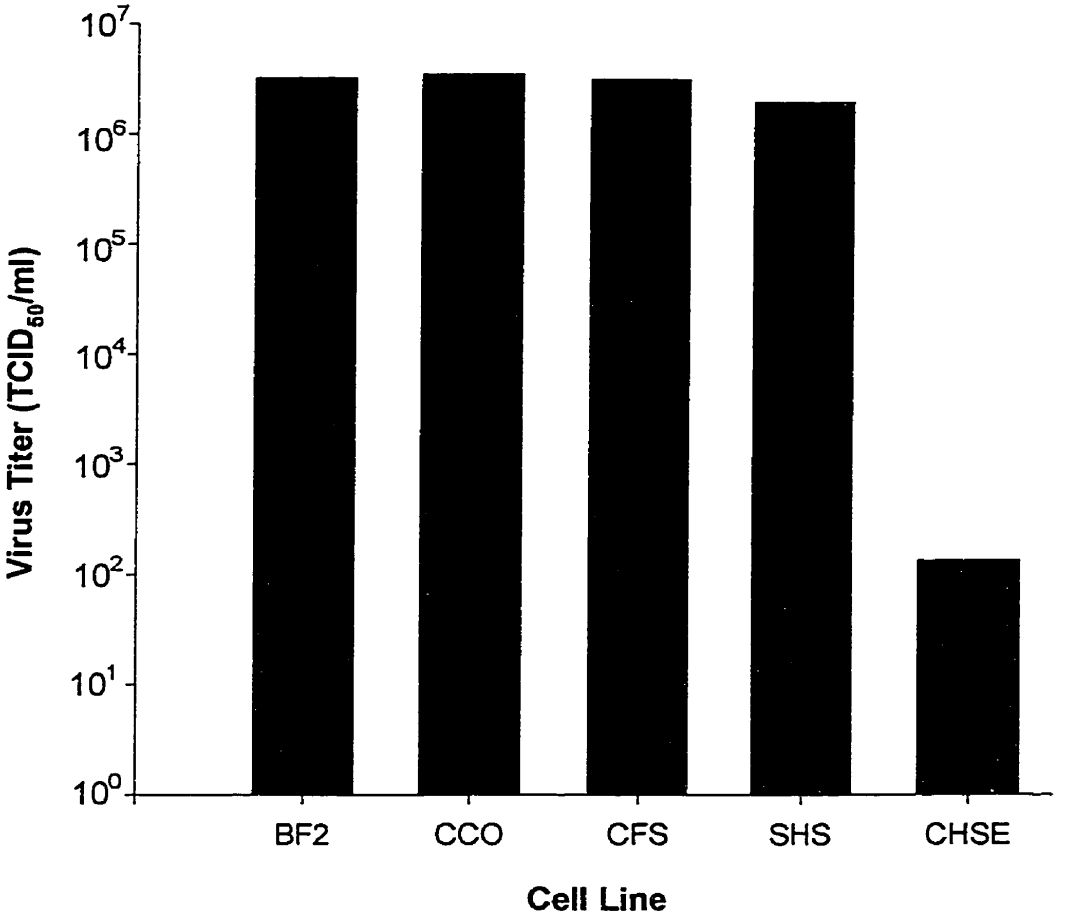

Virus Titer (TCID50/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
BF2
CCO
CFS
SHS
CHSE
Cell Line

Fig. 7.2. Monolayer of catfish spleen cells (CFS) cultured in Leibovitz medium (a) and CFS cells showing cytopathic effects due to the 91-97 virus isolate at 25 °C (b); monolayer of snakehead spleen cells (SHS) cultured in Leibovitz medium (c) and SHS cells with cytopathic effects caused by the 91-97 virus isolate at 25 °C (d).

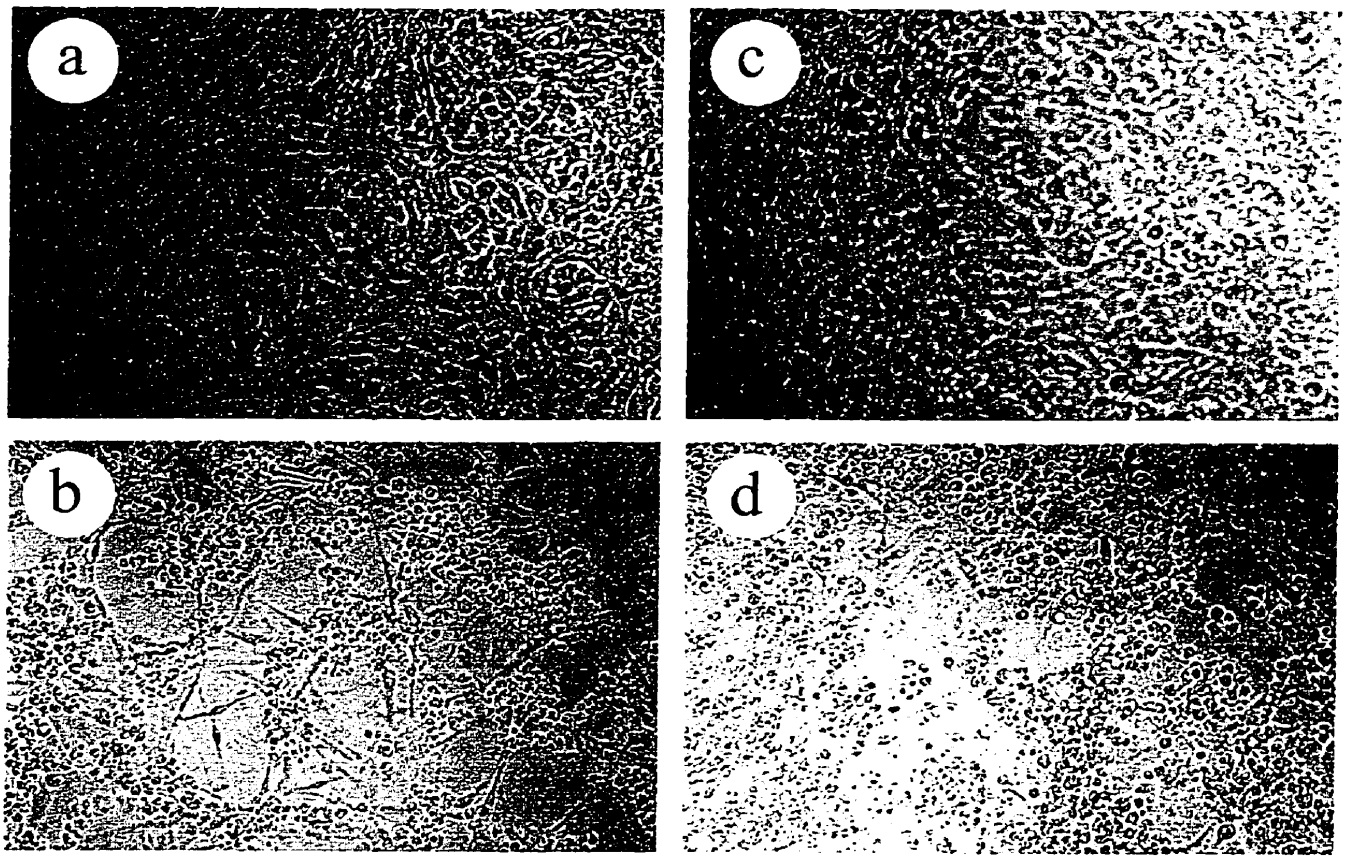
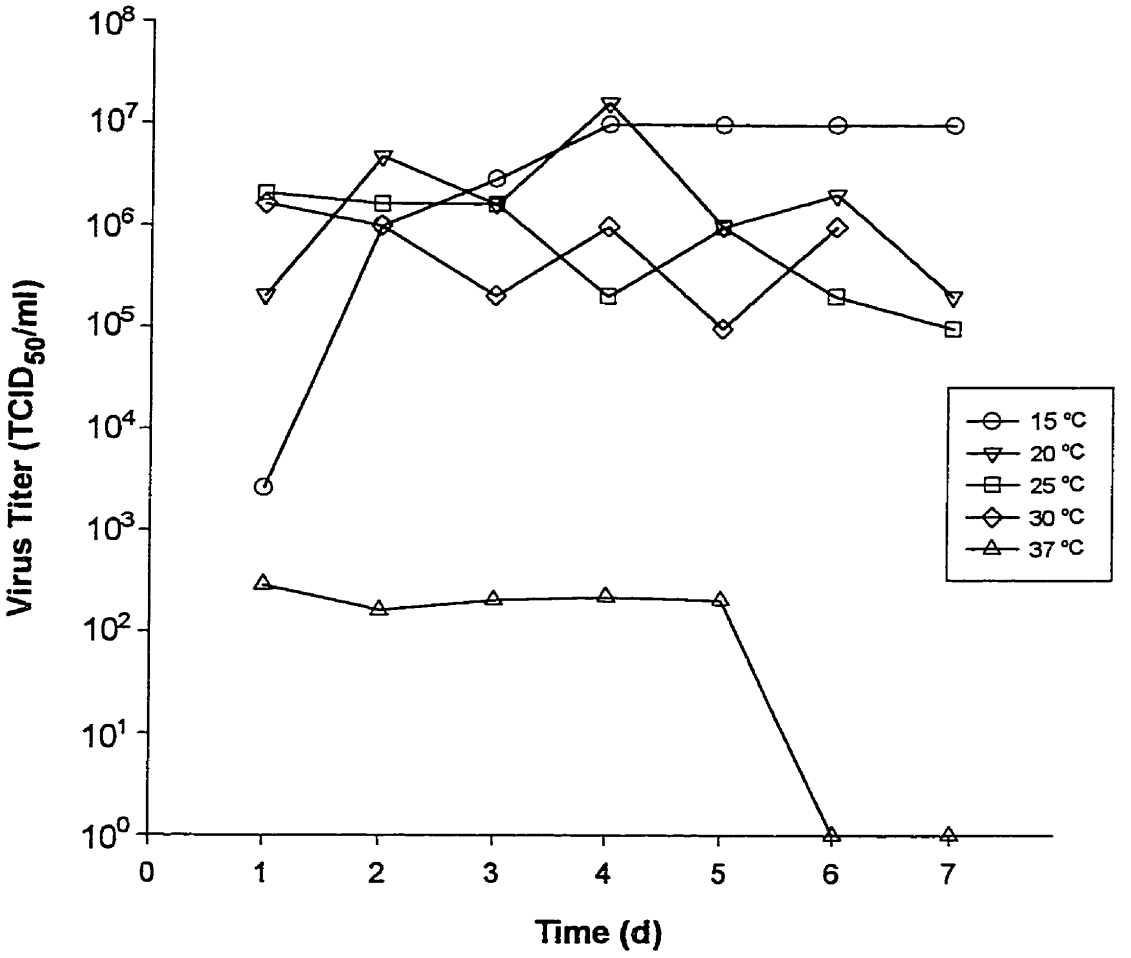

a
c
b
d

Fig. 7.3. Titers of the 91-97 virus isolate in snakehead spleen cells (SHS) following incubation in Leibovitz medium at different times (d) and temperatures (°C).


Virus Titer (TCID50/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
1
2
3
4
5
6
7
Time (d)
15 °C
20 °C
25 °C
30 °C
37 °C

Without the SHS cells, the 91-97 virus' viability can be maintained at -10 °C for 30 d. At 8 °C, the virus remains stable for 10 d while at 20 °C, virus titer was drastically reduced after 3 d. Storage of the virus at 30 °C caused a sudden drop in viability within 3 d of exposure and total loss of the virus at d 6 (Fig. 7.4).

Assay of the cell-free virus (s) and the total virus (c) showed that viral replication followed a typical pattern (Fig. 7.5). 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 one 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. In addition, freezing and thawing the virus caused an approximately half log drop in the titer of the 91-97 virus titer (Fig. 7.6).

Table 7.1 summarizes the effect of several chemical and physical agents on the virus. Exposure to chloroform and heat at 56 °C for 30 min inactivated the virus. Suspension in medium at pH 3 for 30 min caused a loss of viral infectivity of more than two orders of magnitude. IUDR did not affect the virus indicating that it had an RNA genome. Results of the neutralization test using anti-91-97 virus serum showed a cross-reaction with IHNV. Also, the anti-UDRV antiserum cross-reacted with the 91-97 virus isolate (Table 7.2). SHRV did not cross-react with either of the test antisera.

Fig. 7.7 illustrates the 91-97 virus isolate as viewed by transmission electron microscopy (TEM) of SHS cells infected with the virus. The morphology of the virus is typical of a bullet-shaped rhabdovirus with an estimated size of 65 x 175 nm.

Fig. 7.4. Temporal stability of the 91-97 virus isolate at four different temperatures (°C).

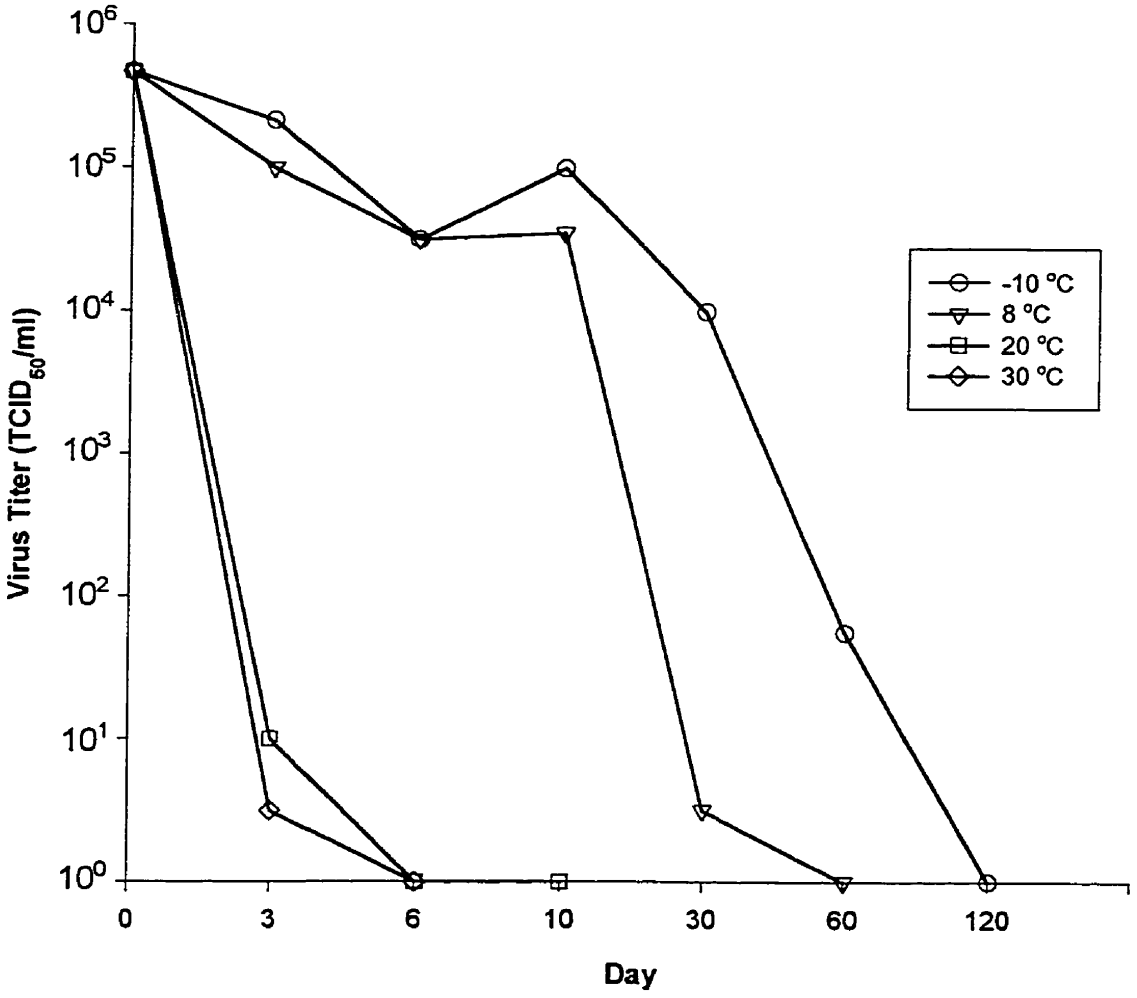

10^6
10^5
10^4
10^3
10^2
10^1
10^0
Virus Titer ($TCID_{50}$/ml)
0
3
6
10
30
60
120
Day
-10 °C
8 °C
20 °C
30 °C

Fig. 7.5. Titers of cell-free (s) and total 91-97 virus isolate (c) in snakehead spleen cells (SHS) incubated for 24 h at 25 °C.

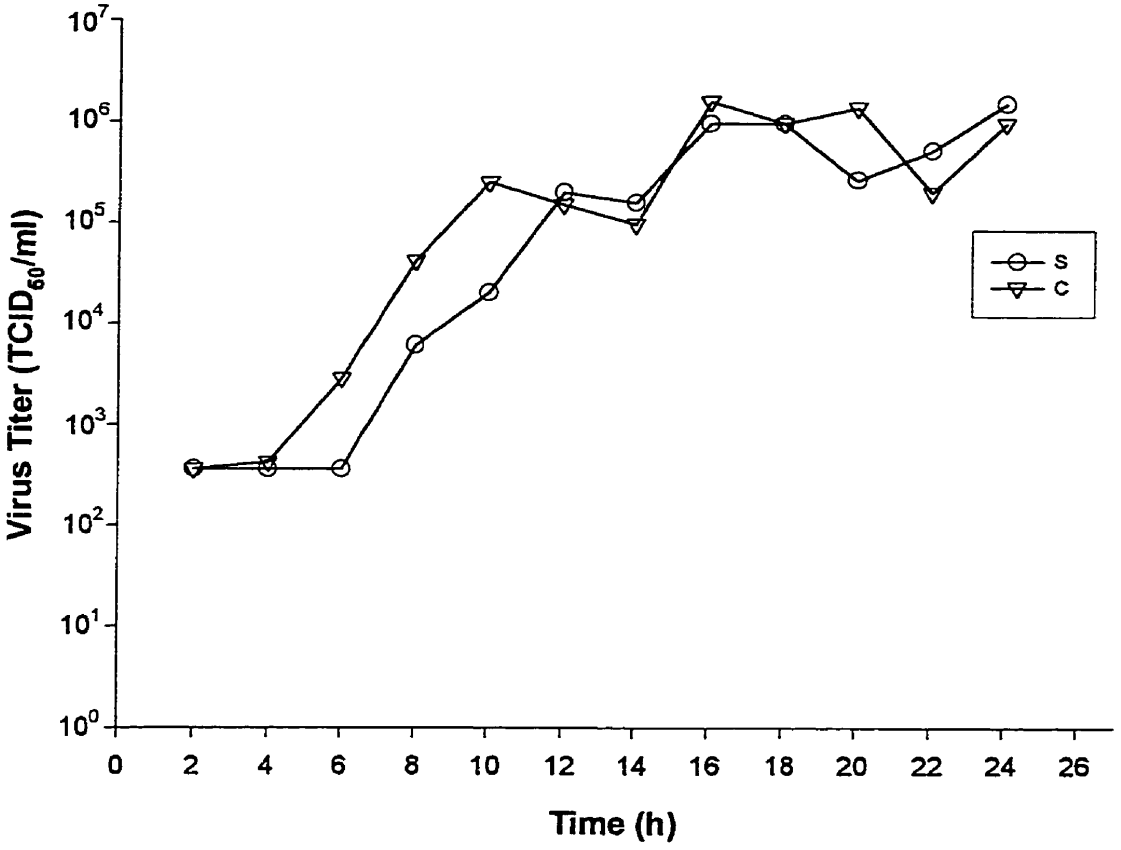

Virus Titer (TCID60/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (h)
S
C

Fig. 7.6. Effect of three freeze-thaw cycles at 1 d intervals on the titer of the 91-97 virus isolate in snakehead spleen cells (SHS) incubated at 25 °C.

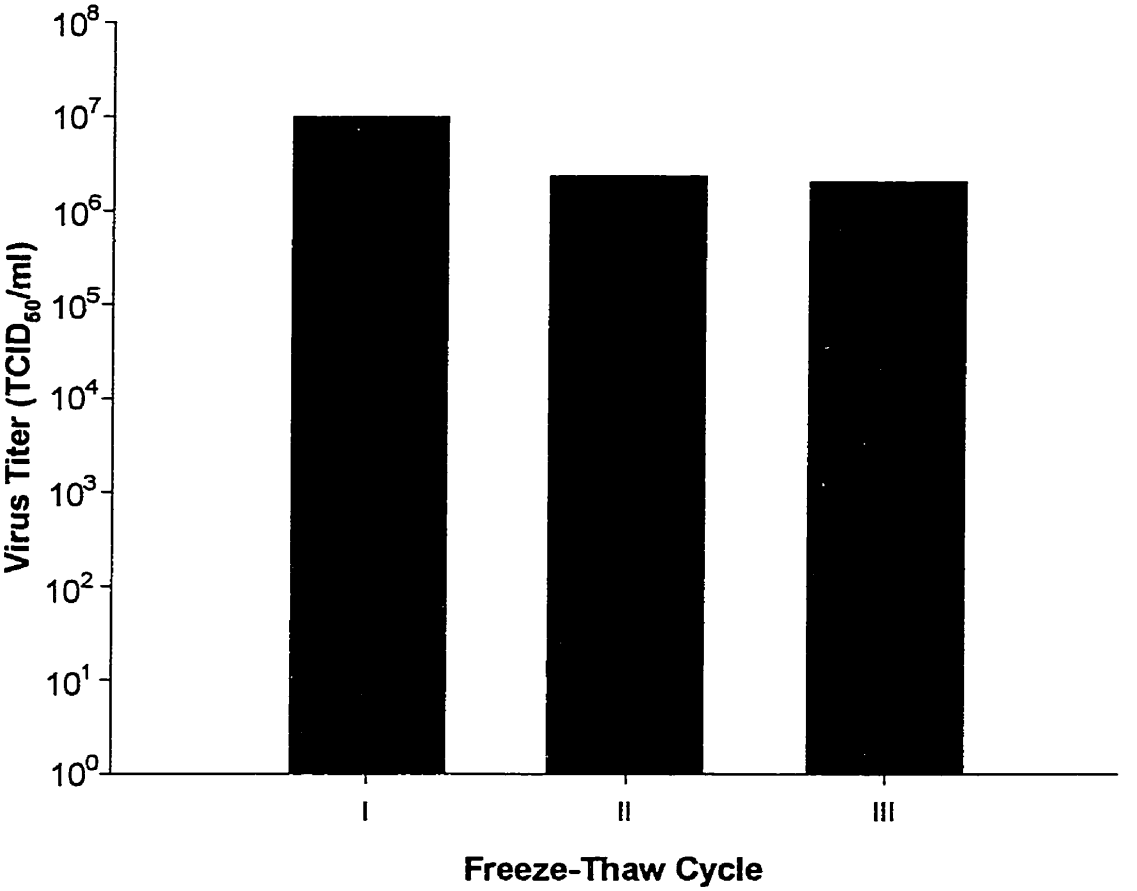

10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Virus Titer ($TCID_{60}$/ml)
I
II
III
Freeze-Thaw Cycle

Table 7.1. Effect of several chemical agents and heat on inactivation of the 91-97 EUS virus isolate from EUS-affected snakehead (*Ophicephalus striatus*).

Agent	Inactivation
Chloroform	yes
Heat at 56 °C (30 min)	yes
IUDR	no
pH 7	no
pH 3	yes

IUDR 5-iododeoxyuridine

Table 7.2. Neutralization titers of several polyvalent antisera with their homologous and other heterologous fish rhabdoviruses.

Virus	Antisera	
	91-97 Virus	UDRV
91-97	91	25
95-96	13	0
UDRV	NT	NT
SHRV	0	0
IHNV	6	NT

91-97	virus isolated from EUS-lesioned snakehead in the Philippines
95-96	virus isolated from EUS-lesioned snakehead in the Philippines
UDRV	Ulcerative Disease Rhabdovirus
SHRV	Snakehead Rhabdovirus
IHNV	Infectious Hematopoietic Necrosis
NT	Not tested

Fig. 7.7. 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 (x77,000).

No observable pathology developed in any of the test fishes following any of the challenges. Although deaths were observed during the initial experiments, they did not occur consistently in subsequent trials. However, virus was detected in visceral organs until d 8 post challenge for fish infected by the IP route and until d 5 for fish infected by other test routes.

The pathogenicity of the virus did not increase after passage in snakeheads (*O. striatus*). Challenges with the virus passed in fish were evaluated by the presence of virus in liver, spleen, gonad, kidney and gills and virus was isolated from gonads (67 - 100 %), kidneys (33 - 66 %) and livers (0 - 33 %). Spleens and gill filtrates also yielded positive results after blind passages.

DISCUSSION

This is the first record of a virus isolated from fish in the Philippines. The 91-97 virus was sensitive to heat at 56 °C, pH 3 and choroform, 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 shape places it in the Family Rhabdoviridae.

The 91-97 virus was propagated in a wide range of cells, such as CFS, SHS, BF2 and CCO. Growth in CHSE cells was very minimal while AS, EPC and RTG-2 were refractory to the 91-97 virus. Thus the actual rate of virus maturation and release from infected cells was dependent on the type of cell line used.

The 91-97 virus replicated rapidly in SHS cells. Incubation time from initial assembly to release from cells was as fast as 4 and 8 h, respectively, which indicates a logarithmic growth rate and a typical viral release pattern. A similar observation was reported with the SHRV rhabdovirus isolated from EUS-affected fish in Thailand (Kasornchandra *et al.* 1991).

Optimum growth producing viral titers of 10^5 to 10^7 $TCID_{50}$ per ml occurred over a wide temperature range of 15 to 30 °C. While replication was initially most rapid at 25 and 30 °C, the highest titer of 2.13 x 10^7 $TCID_{50}$ per ml was achieved at 15 °C by d 4. The same observations were reported for UDRV (Frerichs *et al.* 1989b). The replication temperature requirements corresponded with the water temperature ranges occurring during the EUS outbreaks. In addition, the 91-97 virus tolerated incubation at 37 °C for 5 d but was inactivated by d 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. Under natural conditions, it is likely that the virus is quickly inactivated in the aquatic environment when exposed to temperatures above 30 °C.

In developing countries, such as the Philippines, where electrical outages are common, 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 91-97 virus at higher temperatures of -10, 8, 20 °C should not exceed 30, 10 and 3 d, respectively. These data also serve as a useful guide to temperatures needed during sampling and transport of the virus.

Serological tests showed the 91-97 virus antiserum cross-reacting with UDRV and IHNV but not to SHRV. This indicates that the 91-97 virus isolate is different from SHRV and possibly related to UDRV. Other workers have also shown the unrelatedness of UDRV to IHNV (Kasornchandra *et al.* 1992).

A birnavirus (Hedrick *et al.* 1986), several rhabdoviruses (Frerichs *et al.* 1989a; Kasornchandra *et al.* 1991; Lilley and Frerichs 1994) and a reovirus (Roberts *et al.* 1994) have been previously 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 since this disease was first reported. Except for the 91-97 virus which had a 35 % recovery rate (Chapter 6), previous isolations of rhabdoviruses from fish showing EUS have been inconsistent and often below 5 % (Frerichs 1994). Present results, however, show the importance of rhabdoviruses as potential primary agents of EUS.

Isolations of rhabdoviruses from EUS-affected fish in other Asian countries include seven isolates (BPV, 02, 19, 20E, A4, SL11 and T9204) studied by Lilley (1994). The first six rhabdoviruses appear to form a structurally homogenous and antigenically related group known as UDRV while the latter virus (T9204) is morphologically and antigenically different and may be similar to the SHRV described by Kasornchandra *et al.* (1991) and Lilley and Frerichs (1994). Based on morphological, cytopathological and serological differences, Kasornchandra *et al.* (1992) characterized the genus of SHRV as lyssavirus and the UDRV as vesiculovirus. In the 1993-94 EUS outbreak in Thailand, nine rhabdovirus strains were isolated depicting geographical differences of the lyssavirus (serotype Sh) and of the vesiculovirus (serotype Ud) isolates (Kanchannakhan 1996).

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. SHRV replicates in EPC while UDRV and the 91-97 virus do not. A comparison of the morphological, growth, biochemical and cytopathological characteristics of the 91-97 virus and the antigenic relationships obtained from the present study indicate strong similarities with the UDRV. Further studies are required to define other similarities or differences. It is proposed here that the 91-97 rhabdovirus be delineated by the name EUS rhabdovirus (ERV).

Results of these experiments indicate that ERV is not pathogenic to snakeheads. Fish passage of the virus did not enhance virulence of the virus. Similar observations have been reported by Frerichs *et al.* (1993) using snakehead fish rhabdovirus and the snakehead cell line retrovirus by intraperitoneal injection and by bath.

Attenuation of ERV and the rhabdovirus isolated by Frerichs *et al.* (1986) is possible due to the numerous passages of the virus in cell culture prior to the pathogenicity experiments. Attenuation was suggested when pike fry rhabdovirus reference strain failed to induce pathologic changes/effects in pike fry (Haenen and Davidse 1993).

ERV was recovered from fish visceral organs until d 8 post-inoculation for IP injected virus and d 5 for other test routes of the virus which indicated that the virus remained viable in the inoculated fish for a period of time. That the virus was frequently isolated from the gonad (67 - 100 %), kidney (33 – 66 %) and liver (0 - 33 %) and at lower titers in the spleen and the gill suggests the predilection of the test virus to these

organs. Thus, the role of viruses in the epizootiology of EUS, based upon experiments using the 91-97 isolate, remains uncertain.

CHAPTER 8

HORIZONTAL TRANSMISSION OF THE EPIZOOTIC ULCERATIVE SYNDROME (EUS) IN CATFISH (*CLARIAS BATRACHUS*) AND SNAKEHEADS (*OPHICEPHALUS STRIATUS*) UNDER SIMULATED NATURAL CONDITIONS

INTRODUCTION

Previous studies demonstrated the presence of a rhabdovirus (91-97) among fishes affected with EUS in the Philippines (Chapter 6). The virus was subsequently characterized and named as ERV (Chapter 7). In Thailand, Myanmar and Sri Lanka, the ulcerative disease rhabdovirus (UDRV) and the snakehead rhabdovirus (SHRV) were, likewise isolated from EUS-affected snakeheads and eels (Ahne *et al.* 1988; Frerichs *et al.* 1986; Kasornchandra *et al.* 1992; Lilley and Frerichs 1994). Infectivity trials using ERV failed to establish its pathogenicity to snakeheads (*Ophicephalus striatus*) perhaps because of viral attenuation (Chapter 7). Similar negative results were obtained by Frerichs *et al.* (1993).

Studies on horizontal transmission of EUS under natural conditions have consistently shown that the disease can be naturally transmitted (Balasuriya *et al.* 1990;

Cruz-Lacierda and Shariff 1995). However, transmission of any associated microbe was not demonstrated since microbial isolations were not carried out.

The objectives of this portion of the study were to investigate (1) the horizontal transmission of EUS among catfish and snakeheads under natural conditions and (2) the possible horizontal transfer of ERV from diseased to healthy fish.

MATERIALS AND METHODS

Experimental Fish

Healthy, naive catfish, 20 - 35 g, and snakeheads, 30 - 80 g, were collected from selected rivers in Iloilo and transported to the Tigbauan Station of the Southeast Asian Fisheries Development Center (SEAFDEC). The fish were acclimated at ambient temperature (28 – 32 °C) for at least 1 week in 1000-liter fiberglass tanks before being transported by air to the SEAFDEC Binangonan Freshwater Station in Laguna de Bay. The water in which the fish were transported contained 10 mg per L tricaine methane sulfonate (MS222) during transport. At the Binangonan Station, the fishes were again acclimated in separate 1.5 x 3 x 2 m concrete tanks with fresh aquifer water for 1 week before the experiments.

Catfish Treatments

Five healthy, naive catfish were stocked in each of three epoxy-painted aluminum tanks (1 x 1.5 x 0.5 m), each of which was half-filled with rearing water and covered with

a fish net. Treatments consisted of catfish cohabited with an equal number of EUS-affected snakeheads in lakewater (I), catfish in lakewater (II), and catfish in aquifer water (III). The experiment was conducted in early February - March 1993, the EUS season.

Snakehead Treatments

Twenty healthy, naive snakeheads were placed in each of four replicate net cages (1 x 1 x 1 m) suspended in four separate concrete tanks (3 x 3 x 1.5 m) containing aquifer water (A), or lakewater (B), or cohabited with EUS-affected fish in lakewater (C), or with apparently healthy snakehead in lakewater (D). After termination of the experiments, the tanks were chlorinated and thoroughly rinsed with aquifer water. Two additional treatments were set up consisting of 10 naive snakeheads per cage in 2 replicates with EUS-affected snakeheads in aquifer water (E) or with apparently healthy snakeheads in aquifer water (F). The experiments were conducted for 15 - 40 d.

The tanks were filled with water the day before the experiment while the EUS-affected and apparently healthy fish were placed 2 h before stocking of the naive fish. One EUS-affected or apparently healthy fish, between 250 - 900 g, was placed in each of eight smaller net cages (0.3 x 0.5 x 0.5 m) immediately adjacent to the larger cages containing the naive fish. Lakewater, EUS-affected fish and apparently healthy snakeheads were taken from Laguna de Bay at the peak of the December - February 1994-95 EUS outbreak. Naturally EUS-lesioned snakeheads that died after stocking in the smaller net cages were replaced during the first week of the experiments.

Monitoring of Experiments

All tanks/cages were checked daily for fish mortality and development of EUS lesions (morbidity) according to the criteria in Table 6.1. Mortalities among naturally EUS-affected and apparently healthy snakeheads were also monitored. Water temperature was recorded twice daily, at 0800 and 1700 h. Fish were fed with previously steamed and cooled marine fish.

Tissue Sampling for Virus Detection

Experimental fish were sampled on 0, 10, 20, 25 and 40 d following initiation of the trial, as applicable, when fish were available upon the sampling date. Each fish was anaesthetized in MS222 and disinfected externally with a cotton swab soaked in 70 % ethanol before dissection. Primary isolation of the virus was made from tissues excised from lesions/muscle, liver, gonad, kidney, spleen and gills of the naive snakeheads, EUS-affected fish, fish with healing EUS lesions, and apparently healthy fish. When organs were small, samples from two fish were pooled.

Each tissue sample was processed as described in Chapter 6 and the tissue filtrates stored at -70 °C until viral assay.

Tissue Culture and Viral Assay

Channel catfish ovary (CCO) cells were cultured in MEM-10 in tissue culture flasks (Falcon) at 25 °C. For primary virus isolation, the cells were seeded into 24-well plates (Falcon) with MEM plus 4 % FBS (MEM-4). After 24-h incubation, cells were fully confluent and the medium was aseptically removed. Then 100 ul of each filtrate was

gently overlaid onto the cell monolayer. The plates were rocked gently for a few seconds and then incubated for 1 h at 22 - 25 °C after which each well was inoculated with one ml MEM-4 buffered with 1M N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid (HEPES). The plates were then incubated at 25 °C for 7 d and examined microscopically daily for CPE. Blind passages were carried out on negative samples at least 5 times. Only those isolates manifesting consistent CPE in all subsequent passages were considered positive for virus. GIBCO media, reagents and antibiotics were used and prepared as described in Chapter 6.

Statistical Tests

Morbidity and mortality data in the snakehead experiment were processed using the repeated measures analysis of variance and significance determined using Duncan's multiple range test.

RESULTS

In naive catfish cohabited with EUS-affected snakeheads in lakewater, EUS-like lesions initially developed by 13 d. These subsequently developed to moderate lesions by d 15 (Table 8.1) and progressed to severe lesions by d 18 - 21. In contrast, naive catfish reared in either lake or aquifer water alone did not develop any lesions even until d 25 when the experiment was terminated. The temperature during the experiment ranged from 23 - 26.5 °C (0800 h) and 26 - 29 °C (1700 h) (Fig. 8.1.).

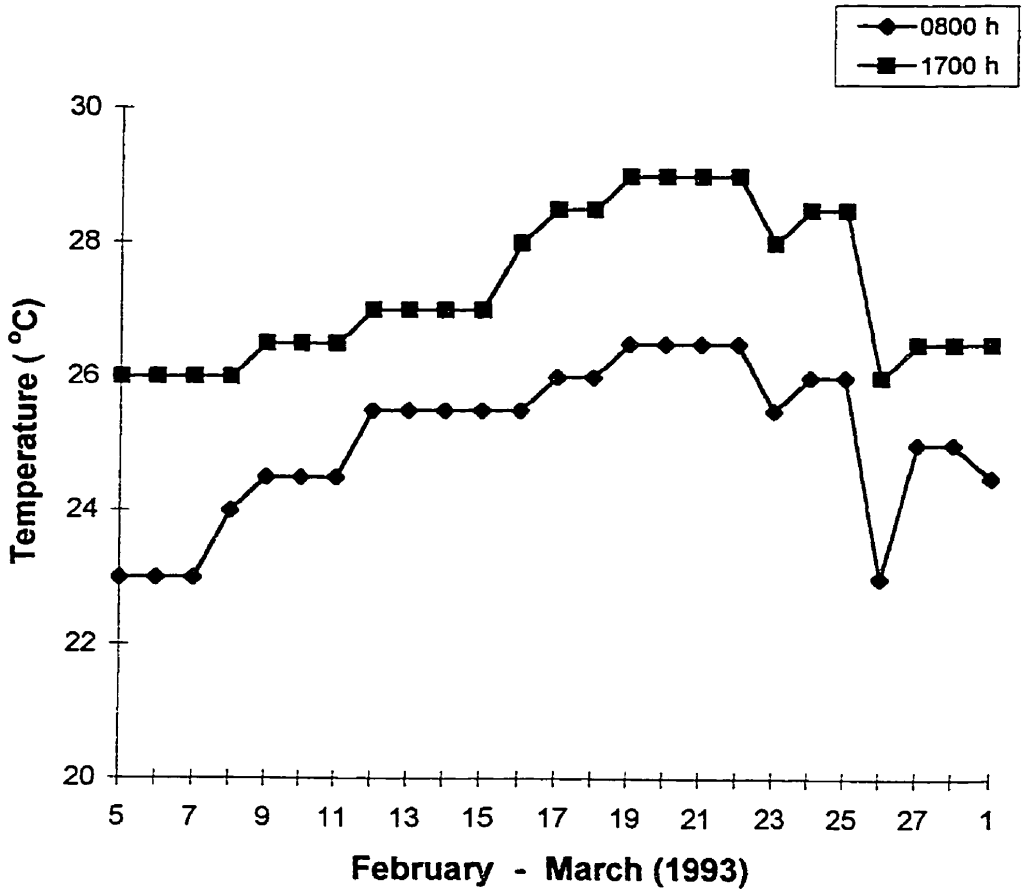
Table 8.1. Lesion development in naive catfish (*Clarias batrachus*) cohabiting with EUS-affected snakeheads (*Ophicephalus striatus*) in Laguna de Bay lakewater and aquifer water at a time when epizootics of EUS occur.

Treatment	Day Post-exposure	Lesion (no. of fish)
Catfish + Naturally	13	3-*2+
EUS-affected	14	3-2++
snakeheads in	15	3-2+++
lakewater **	18	1-2+2++++
	21	1-2+2++++
	25	1-2+2++++
Catfish in lake water**	25	5-
Catfish in aquifer water	25	5-

* see table 2.2 for lesion grade criteria

** virus isolated from 1 of 5 EUS-exposed naive fish samples and from 1 of 7 EUS fish

Fig. 8.1. Water temperatures during epizootic ulcerative syndrome (EUS) transmission experiments with catfish (*Clarias batrachus*).


0800 h
1700 h
30
28
26
24
22
20
Temperature (°C)
5
7
9
11
13
15
17
19
21
23
25
27
1
February - March (1993)

Virus was isolated from one of five fish sampled from the EUS+lakewater treatment. Similar results were also obtained from catfish in the lakewater rearing treatment. No virus was isolated from the catfish reared in aquifer water. Two of five EUS-affected snakeheads stocked with the catfish were positive for virus.

Observations on EUS lesion development in the snakeheads placed in experimental cages are summarized in Fig. 8.2. Where naive snakeheads were exposed to naturally affected EUS snakeheads in lakewater (Treatment C) or with apparently healthy snakeheads in lakewater (Treatment D), EUS lesion development was most rapid. Onset of lesions was observed on d 6 (C) and d 7 (D) with 100 % morbidity at d 12 (C) and 14 (D) post exposure (Fig. 8.3). In lakewater alone (Treatment B), onset of the disease was slower with the first lesions occurring at d 16 with 100 % morbidity at d 21 post exposure. Naive fish in aquifer water with naturally affected EUS fish (Treatment E) and in aquifer water with apparently healthy snakeheads (F) manifested EUS lesions on d 20 (F) and 7 (E). Naive fish stocked in aquifer water alone (Treatment A) did not develop EUS-like lesions. Differences among treatments on rates of lesion development and on days post-exposure were highly significant ($p<0.01$). The time and number of naïve fish developing EUS-like lesions in treatments C and D were significantly faster and higher than in the other treatments at d 10 post-exposure. Also, the morbidity pattern observed in treatment B was statistically different ($p<0.05$) from treatments E, F and A, on d 20.

EUS signs began as grade I lesions then gradually advanced to grade III - IV in d 3 to 5 from onset. These periods of lesion advancement were consistently the same for treatments B, C and D (Table 8.2). Gross pathology of the lesions was similar to that of the naturally affected EUS fish with single or, more often, multiple lesions and ulcers or,

Fig. 8.2. Lesion development among 20 naive snakeheads (*Ophicephalus striatus*) each stocked in aquifer water (A); lakewater (B); lakewater with epizootic ulcerative syndrome (EUS) fish (C); lakewater with apparently healthy fish (D); aquifer water with EUS fish (E) and aquifer water with apparently healthy fish (F).

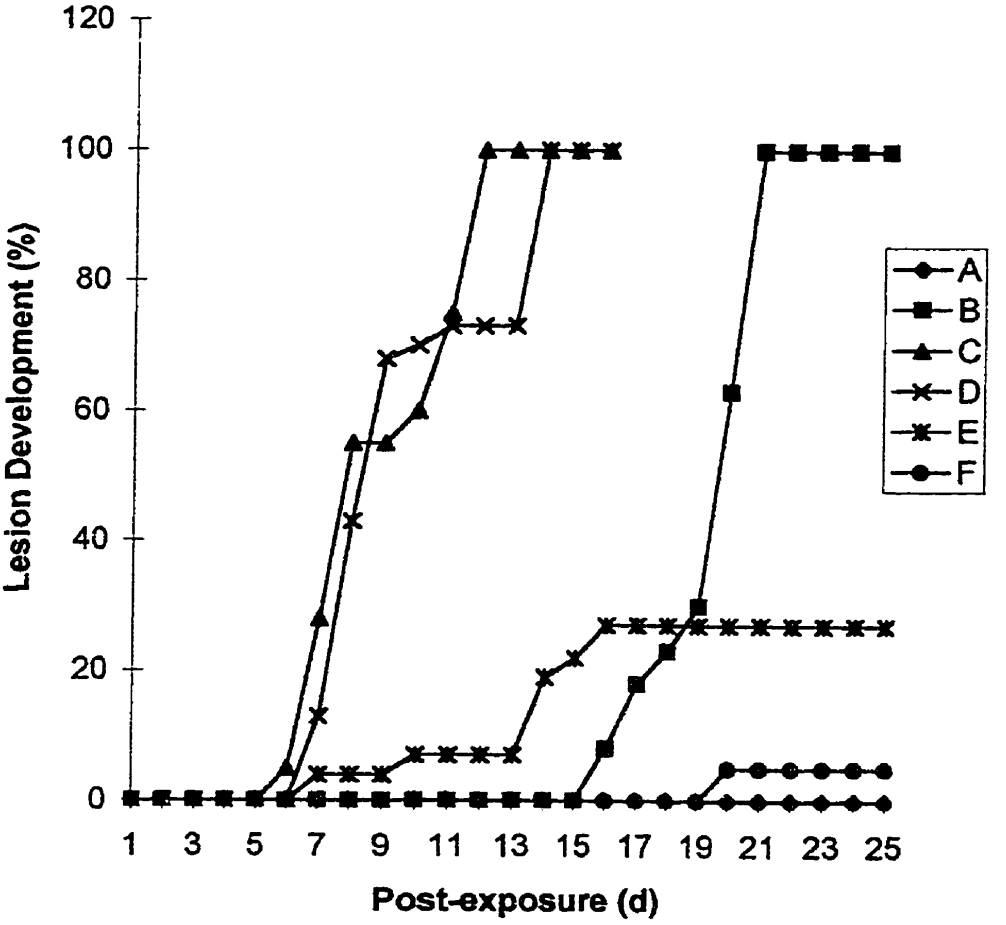

120
100
80
60
40
20
0
Lesion Development (%)
1 3 5 7 9 11 13 15 17 19 21 23 25
Post-exposure (d)
A
B
C
D
E
F

Fig. 8.3. Snakeheads (*Ophicephalus striatus*) showing epizootic ulcerative syndrome (EUS)-like lesions following exposure to lakewater with EUS-affected snakeheads (a) and to lakewater with apparently healthy fish (b).

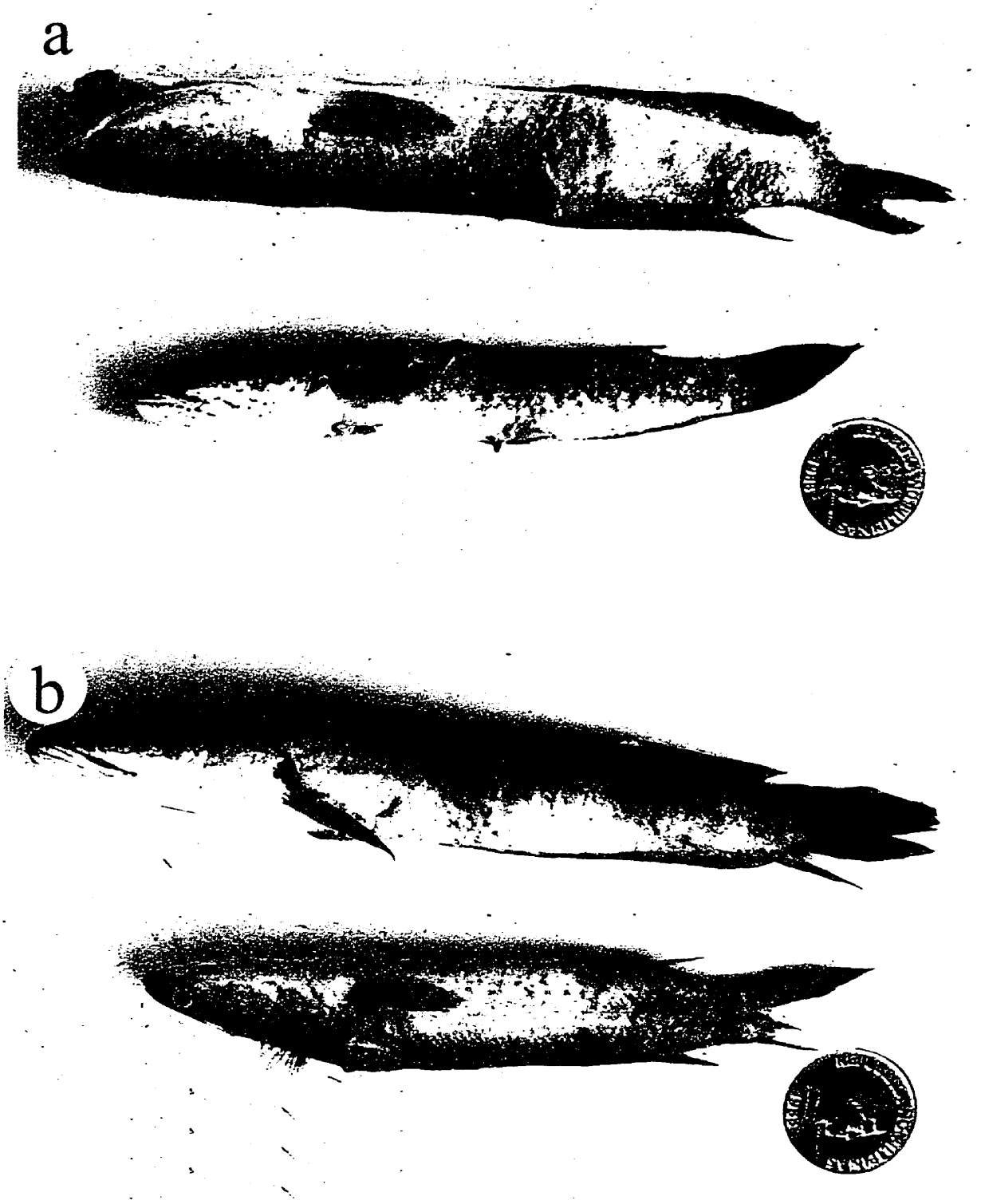

a
b

Table 8.2. Summary of observations on the onset of epizootic ulcerative syndrome (EUS) lesion development and subsequent onset of mortality among naive snakeheads (*Ophicephalus striatus*) following various treatments as described in Fig. 8.2.

Cage	Onset of Initial lesion	Onset of severe lesions from initial lesion detection	Death after onset of moderate lesions
A	no	no	no
B	day 16 - 17	4 - 5 days	6 days
C	day 6 - 7	5 - 6 days	5 - 6 days
D	day 7	5 days	5 - 6 days
E	day 14	no	3 days
F	day 20	no	no

no lesions not observed

more often, multiple lesions and ulcers developing in the head, caudal, dorsal, ventral and or lateral musculature. There was no common pattern observed as to sequence or preferential anatomical site of lesion development. One snakehead in treatment D developed an apparent fungal infection at the lesion site. Several snakeheads in treatments C and D exhibited very pale gills upon sampling. In all experimental samples including EUS-affected and apparently healthy snakeheads, EUS lesion development occurred in both male and female samples, including several gravid fish.

Mortality patterns were most acute in treatments B, C and D (Fig. 8.4). However, mortalities in treatment B were manifested later than those in C and D. Mortalities associated with treatments E and F were gradual and occurred earlier than C and D. Naive fish in treatment A showed the least number of deaths. Initial mortalities among experimentally-infected fish in treatments B, C and D were consistently observed on d 5 - 6 after onset with more than 50 % of the deaths occurring 8 d after onset. The mortality differences among treatments and in time of occurrence were highly significant ($p<0.01$). Mortalities in treatments C, D and E on d 15 and 20 were significantly higher than in other treatments.

For treatments A, B, C and D, the water temperature ranged from 23 - 24.5 °C (0800 h) and 24 - 25 °C (1700 h) during the first 6 d, then 24 - 25 °C (0800 h) and 24 - 28 °C (1700 h) until d 19 (Fig. 8.5). Thereafter, temperatures fluctuated from 24 28.5 °C (0800 h) and 26 - 32 °C (1700 h). During experiments with treatments E and F, temperature ranges were 24 - 24.5 °C (0800 h) and 26 - 27.5 °C (1700 h) in the first 5 d which oscillated between 24.5 - 28.5 °C (0800 h) and between 28 – 30 °C (1700 h).

Fig. 8.4. Cumulative mortalities among 20 naive snakeheads (*Ophicephalus striatus*) stocked in each of aquifer water (A); lakewater (B); lakewater with epizootic ulcerative syndrome (EUS) fish (C); lakewater with apparently healthy fish (D); aquifer water with EUS fish (E) and aquifer water with apparently healthy fish (F).

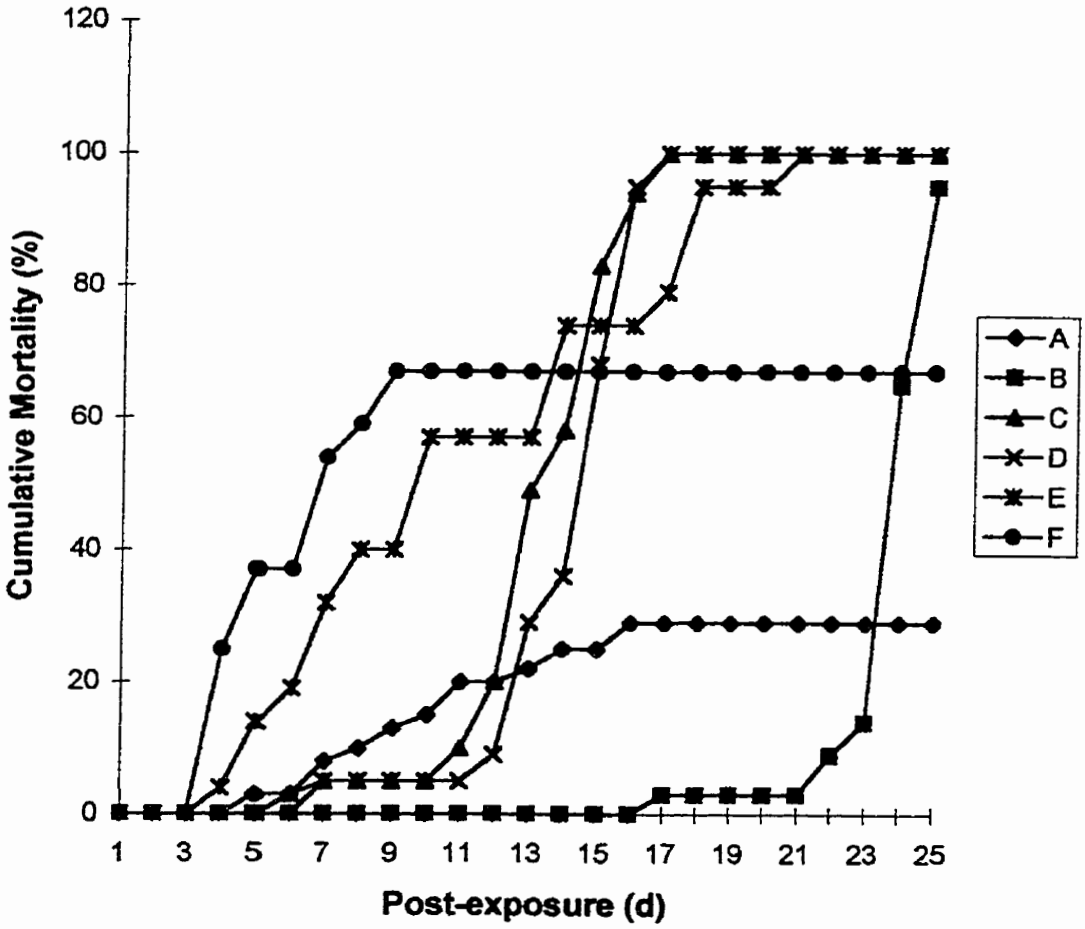
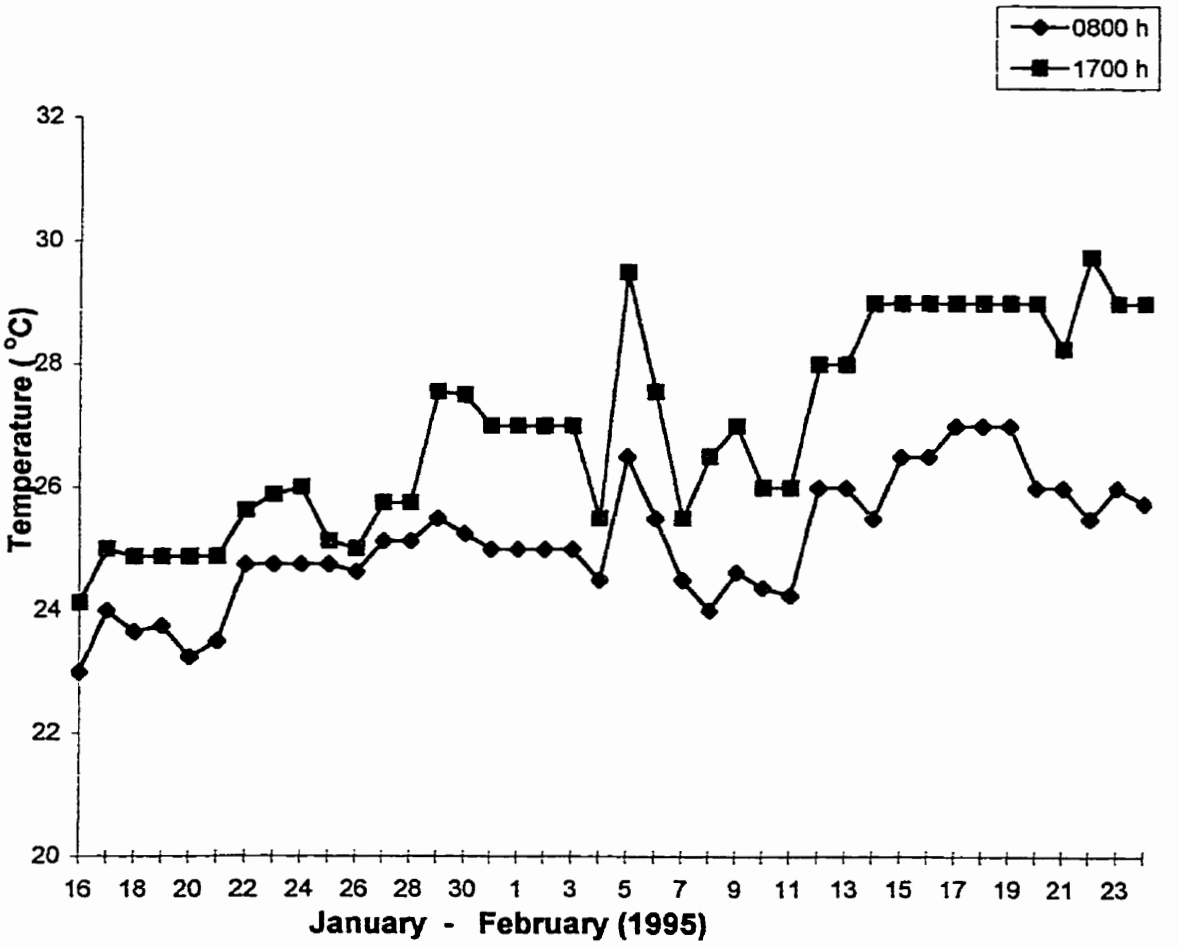

120
100
80
60
40
20
0
Cumulative Mortality (%)
1
3
5
7
9
11
13
15
17
19
21
23
25
Post-exposure (d)
A
B
C
D
E
F

Fig. 8.5. Water temperatures during epizootic ulcerative syndrome (EUS) transmission experiments with snakeheads (*Ophicephalus striatus*).


0800 h
1700 h
Temperature (°C)
32
30
28
26
24
22
20
16 18 20 22 24 26 28 30 1 3 5 7 9 11 13 15 17 19 21 23
January - February (1995)

Mortality occurrences among a total of 74 advanced-lesioned (grades III and IV), naturally EUS-affected snakeheads were observed daily after stocking. Ten percent died within 2 d of stocking, while 45 % died within 4 d of stocking. The greatest number of deaths occurred on d 5 (27 %). Subsequently, fewer deaths were recorded until d 11 post stocking. No mortalities occurred among apparently healthy snakeheads.

The results showed that naive snakeheads were free of virus at the start of the horizontal transmission experiments (Table 8.3). There were no samplings conducted after d 10 in treatments C and D because all experimental fish succumbed to the infection before d 20.

Naturally EUS-affected snakeheads were positive for virus in all of the test organs. Snakeheads with healing lesions were also positive for virus when the liver was used as the sample source. Among apparently healthy snakeheads, however, only 1 of 8 test fish yielded virus. Most organs except livers, were virus positive in this latter case. Among the experimentally treated fish, virus recovery was possible as early as d 10 post-exposure in treatments C, D, E and F. Among snakeheads in treatments A and B, virus was detected on d 20 of the experiment. Virus was recovered in all organs tested with higher success rates from livers, kidneys and spleens than from other tissues. Titer of virus recovered ranged from $10^3 - 10^6$ $TCID_{50}$ per ml tissue filtrate.

DISCUSSION

The almost 100 % frequency with which naive, susceptible test fish acquire EUS

Table 8.3. Isolation of the epizootic ulcerative syndrome (EUS) virus from different organs of several snakeheads (*Ophicephalus striatus*) following different experimental treatments in aquifer water and Laguna de Bay water, at a time when natural epizootics of EUS occur.

Fish Sample	Sample #	Muscle	Liver	Gonad	Kidney	Spleen	Gills
Naive snakeheads	6	-	-	-	-	-	-
Naturally EUS-affected snakeheads	6	2/6(1~2)	4/6(1~4)	1/6(4)	3/6(1~4)	3/6(1~2)	NT
Snakeheads with healing lesion *	2	-	1/2(1)	-	-	-	-
Apparently healthy snakeheads *	8	1/8(1)	-	1/8(1)	1/8(1)	1/8(1)	1/8(1)
A (day 10)	6	-	-	-	-	-	-
(day 20)	6	1/6(4)	2/6(4)	3/6(2~4)	3/6(4)	3/6(2~4)	2/6(3~4)
(day 25)	6	-	-	-	-	-	-
(day 40)	2	-	-	-	-	-	-
B (day 10)	6	-	-	-	-	-	-
(day 20)	6	4/6(3~4)	1/6(3~4)	2/5(2~3)	3/6(3~4)	4/6(3)	3/6(2~4)
(day 25)	2	-	-	-	-	-	-
C (day 10)	6	1/6(4)	2/6(3~5)	1/6(4)	2/6(3~4)	2/6(4)	1/6(4)
D (day 10)	6	-	1/6(5)	0/5	1/6(2)	-	1/6(4)
E (day10)	3	-	1/3(4)	0/2	-	0/2	-
(day 20)	1	-	-	-	-	-	-
F (day 10)	3	1/2(2)	-	0/2	-	-	0/2
(day 20)	2	-	-	1/2(3)	-	-	-

- virus not detected
( ) these numbers refer to passage number at which time the virus CPE was noted
NT not tested
* snakeheads collected from Laguna de Bay
A reared in aquifer water
B reared in lakewater
C cohabitated with EUS fish in lakewater
D cohabitated with apparently healthy fish in lakewater
E cohabitated with EUS fish in aquifer water
F cohabitated with apparently healthy fish in aquifer water

during cohabitation experiments with naturally EUS-affected snakeheads in this study confirmed that horizontal transmission of this disease does occur. These results also provide convincing evidence that EUS infections can be waterborne.

A similar experiment at the same site revealed that the experimentally induced EUS lesions were similar to natural EUS lesions upon histopathological analyses (Cruz-Lacierda 1995). Previous studies using snakeheads also reproduced the EUS lesions (Balasuriya *et al.* 1990; Cruz-Lacierda and Shariff 1995), thus reinforcing the hypothesis that a microbial agent is involved in this disease. The same observations were made by Balasuriya *et al.* (1990) who found advanced lesions developing in d 2 - 3 following onset and mortalities after 8 d. On the other hand, in the study of Cruz-Lacierda and Shariff (1995), onset of EUS lesions among unfed experimental snakeheads were observed at d 9 and 10 while onset among fed snakeheads occurred at d 14. Nevertheless, advanced lesion development also occurred on d 5 - 6 after onset. This is the first report of a successful horizontal EUS transmission in catfish under natural conditions.

Recovery of the virus by tissue culture from experimentally exposed fishes under simulated natural conditions is a new finding. Moreover, titer of virus recovered ranged from $10^3 - 10^6$ $TCID_{50}$ per ml tissue filtrate. These results strongly suggest that the virus is implicated in the pathogenesis of EUS. In Thailand, virus-like particles were found in the spleen, ciliary endothelium and muscle fibrils of snakeheads during epizootics (Wattanavijarn *et al.* 1983).

Results reported here are the first to show that apparently healthy fish are capable of transmitting ERV to naive fish. The onset of signs of EUS at d 6 or 7 was most rapid in naive fish exposed to either naturally affected EUS fish or to apparently healthy fish. The

almost similar morbidity and mortality rates and trends observed in treatments C and D (Fig. 8.2 and 8.3) implies that apparently healthy fish shed virus at about the same rate as the EUS-affected fish. The present study strongly suggests that at least some apparently healthy fish found in EUS-endemic sites are carriers of ERV. In waters where EUS positive fish or virus fish carriers abound, they continuously shed virus into the rearing waters.

That lakewater was a medium of transmission of ERV was shown by development of EUS lesions among naive snakeheads exposed to the lakewater. The experiment with catfish was terminated as soon as all fish in the treatment with naturally affected snakeheads manifested signs of EUS. That the catfish in the lakewater treatment had not yet shown positive signs of EUS lesion development indicated that virus titer in the lakewater was probably lower, therefore required a longer incubation period of infected fish to manifest the lesions (Fig. 8.2). This hypothesis was confirmed by the development of EUS lesions among lakewater-exposed naive snakeheads that showed onset of lesions much later (a delay of 10 d). However, virus was also recovered from both naive catfish and snakeheads exposed to only lakewater. In previous studies with similar treatments, onset of lesions was not observed until d 44 (Cruz-Lacierda and Shariff 1995).

The results, likewise, suggest that the aquifer water taken from the nearby EUS-enzootic area carried the ERV and can transmit it to naive fish. However, resulting lesion onset was much delayed or not manifested at all (as in treatment A). It is also possible that a vector was present which contaminated the aquifer water. Previous virus isolations in SHS cells from golden snails that proliferated in EUS-enzootic sites indicated the presence of virus (unpublished data). Further studies should explore the vector question.

In treatments B, C and D, the infection progressed to yield prevalence of 100 % (Fig. 8.2). Close examination of the results showed that the interval between lesion onset to manifestation of severe lesions was about 5 d in treatments B, C and D even though onset in B was not concurrent with that of C and D. Consequently, death from onset was also consistently 5 or 6 d after onset of lesions with more than 50 % mortality occurring 8 d after onset (Fig. 8.3). Hence, the incubation period of EUS may differ depending on the source of viral contamination but the pathogenesis of the disease follows a similar pattern and rate once EUS signs are detected. That mortality can be attributed to EUS was shown by the acute mortality rates following the onset and advanced lesion development among experimental fish in treatments B, C and D. Fish bites might have exacerbated transfer of virus. In addition, the presence of decomposing carcasses (from fish that die during the night or during sampling intervals) may cause an increased release of virus in the water as, hypothesized by Mulcahy *et al.* (1983b).

Natural transmission of EUS appeared to be associated with low water temperatures, which prevailed during the months of the EUS seasonal outbreaks. Since viral infectivity of ERV in a cell-free environment can be reduced after 3 d at 20 °C and drastically affected within 3 d at 30 °C (Chapter 7), this may partially explain why EUS outbreaks predominantly occur during the cooler season in the tropics. Since both male and female fish and even gravid females were found positive for virus, and taking into consideration the isolation of virus from the ovaries of EUS-affected fish, this suggests that vertical transmission of the ERV is possible. This may partially explain the persistence of ERV in fish populations in enzootic sites, which result in the annual outbreak of EUS.

This is a first report on detection of ERV in specific organs of naturally and experimentally infected snakeheads. Previous reports on viral isolations were conducted using pooled tissues (Chapter 7; Frerichs *et al.* 1989b). Since virus was recovered from all test organs, this demonstrates a lack of organ specificity of ERV. It is not clear how the virus enters the target fish. A more detailed study regarding this aspect is needed.

Overall success rate in viral isolations among experimental snakeheads in this study was 33 %, which agrees closely with the 35 % isolation rate observed in the results obtained in Chapter 6. Thus, the threshold of detection of ERV using the 1:10 dilution rate of tissue filtrate method for virus isolation used is further confirmed.

The foregoing results indicate that exposure to contaminated water is a significant factor in the spread of ERV among uninfected fish stocks and that the development of EUS epizootics can occur when naive, uninfected fish are mixed with infected fish. The results strongly suggest the involvement of a virus.

To summarize, the horizontal transmission of the viral pathogen from EUS-affected and apparently healthy snakeheads to naive snakeheads under simulated natural conditions is confirmed. For the first time, virus was recovered from naive fish experimentally exposed to virus-infected fish and waters. It is also the first report on horizontal transmission of EUS between species, i.e. snakeheads to catfish. In addition, water temperature appears to be an important factor in the pathogenesis and transmission of EUS.

CHAPTER 9

THE EPIZOOTIC ULCERATIVE SYNDROME (EUS) VIRUS: EVIDENCE OF *IN-VIVO* PATHOGENICITY TO SNAKEHEADS (*OPHICEPHALUS STRIATUS*)

INTRODUCTION

Frerichs *et al.* (1993) was unable to show that the viruses that they isolated from EUS-diseased fish could cause EUS when injected into healthy fish. Likewise, in Chapter 7, it was shown that the 91-97 virus isolate from EUS-diseased snakeheads did not cause EUS lesions in naive snakeheads. However, in chapter 8, it was demonstrated that the transmission of EUS among catfish and snakeheads occurs under simulated natural conditions. In addition, the results revealed that EUS-affected and apparently healthy fish from EUS-endemic areas harbored viruses. Several viruses were then isolated anew from naive fish exposed to EUS-diseased and from apparently healthy snakeheads obtained from an EUS area.

The purpose of this study was to test the pathogenicity of the newly isolated viruses on naive snakeheads and to establish the possible mode of viral transmission.

MATERIALS AND METHODS

Viral Isolates

Several virus isolates from naturally EUS-affected snakeheads (*O. striatus*) coded 95-1 (integument lesion), 95-2 (liver), 95-4 (kidney), 95-5 (spleen), 95-15 (spleen), and from snakeheads to which EUS had been naturally transmitted and coded 95-91 (gonad), 95-96 (lesion), 95-117 (gills), as described in Chapter 8, were selected and used for the experiments. The bracketed words denote the organs from which the virus was isolated. Virus suspensions were preserved at -70 °C until used.

Tissue Culture and Viral Propagation

Monolayers of CCO cells were grown in MEM-10 and an antibiotic-antimycotic mixture (100 I.U. penicillin G sodium, 100 ug streptomycin sulfate, and 25 ug amphotericin B per ml medium). Cells were seeded in 25-cm^2 tissue culture flasks (Falcon) at 25 °C and then subcultured into 75-cm^2 tissue culture flasks (Falcon).

After 24 h incubation, when cells were fully confluent, the medium was aseptically removed and 1.5 ml of a 10^3 $TCID_{50}$ per ml of each virus suspension was gently overlaid onto the cell monolayer in a 25-cm^2 flask. Inoculated flasks were rocked gently for a few seconds then incubated for 1 h at 22 - 25 °C after which 8 ml MEM with 4 % FBS (MEM-4) were added to each flask. The flasks were incubated at 25 °C for 3 to 5 d or until 75 % of the cells manifested CPE. The supernatant was clarified by centrifugation at 2500 rpm for 15 min at 4 °C. Harvested viruses were stored in aliquots at -70 °C until used.

Viral titers were estimated by the tissue culture infectious dose-50 % end point ($TCID_{50}$) method in 96 well plates using MEM-4 with the antibiotic-antimycotic mixture and buffered with 1M HEPES. The plates were then incubated at 25 °C for 7 d. The plates were examined microscopically for CPE and $TCID_{50}$ estimates computed using the Reed and Muench method (1938) as described by Rovozzo and Burke (1973).

Experimental Fish

Wild, healthy juvenile snakeheads (40 - 80 g), fingerlings (0.93 - 1.21 g), and fry (0.12 - 0.21 g) were collected from several rivers in Iloilo. Since EUS has never been observed in Iloilo and viruses could not be isolated from sampled Iloilo snakeheads in previous studies, these fish were considered naive fish. The fish were acclimated for 1 week in freshwater in 1000-liter fiberglass tanks at ambient temperature and for at least 3 d at 20 - 22.5 °C before initiating infection experiments. When used for experiments, the fish were placed in 5-liter aquaria containing cooled, pre-boiled or UV-sterilized unchlorinated freshwater. For each aquarium, stocking densities were one to two juvenile fish or 10 fingerlings/fry.

Infection Experiments

Naive juvenile snakeheads were injected intramuscularly with 0.1 ml virus isolates 95-1, or 95-4, or 95-96 or with sterile MEM-4 (controls). Viral doses inoculated were 10^5 or 10^6 $TCID_{50}$ per fish. Three replicate aquaria with two fish per aquarium were tested (20 - 22.5 °C rearing water temperature).

Effect of Temperature on Viral Virulence

Two naive juvenile snakeheads per aquarium, stocked at two temperature conditions, 20 - 22.5 °C or 28 - 32 °C, were injected intramuscularly (IM) or intraperitoneally (IP) with virus isolate (95-1) at 10^6 $TCID_{50}$ per fish. MEM-4 was injected into the control fish.

Unpassed Viral Isolate vs Virus Passed 3 or 4 Times

Two naive snakehead juveniles per aquarium were injected intramuscularly with viruses from fresh tissue filtrates (95-1, 95-4, 95-5), obtained from naturally-affected EUS snakeheads and had never been propagated in tissue culture (unpassed) or with the virus of the same coded tissue filtrates that were propagated in CCO cells 3 or 4 times (passed). Unpassed filtrates had $TCID_{50}$ doses of 10^1, 10^4 and 10^3, respectively; while passed viral isolates had $TCID_{50}$ doses of 10^6, 10^5 and 10^7, respectively. MEM-4 was injected into the control fish. The experiment was conducted in rearing water at 20 - 22.5 °C.

Dose Response Experiment

Five naive juvenile snakeheads stocked individually in aquaria were injected IM with the 95-96 virus isolate at doses of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 0 $TCID_{50}$ per fish. The experiment was conducted in rearing water at 20 - 22.5 °C.

Viral Isolates from Different Organ Sources

Virus isolates 95-2 (liver), 95-4 (kidney), 95-15 (spleen), 95-91 (gonad), 95-96 (lesion) and 95-117 (gills) from EUS-affected snakeheads were each injected (IM) into two naive snakehead juveniles per aquarium. MEM-4 was injected into two control fish. The experiment was conducted in rearing water at 20 - 22.5 °C.

Route of Virus Infection

Virus isolates 95-1, 95-4 and 95-96 were used for this experiment. Five naive snakeheads per treatment were each exposed to the virus isolates separately by IM injection, IP injection, intubation, subdermal (SD) injection, and by bath at 20 - 22.5 °C. Injection and intubation treatments consisted of a 0.1 ml dose of the virus inoculum or 10^6 $TCID_{50}$ per fish. However, in the subdermal treatment, scales were removed over a 1-cm^2 area using the blunt top of a sterile scalpel blade. At the center of the de-scaled area, a slit 0.5 cm long x 0.25 cm deep was made into which 1.0 ml viral suspension was placed. Bath treatment was carried out by immersion of naive fish in water containing virus (10^5 $TCID_{50}$ per ml) for 1 h before increasing the water volume to 5 liters (a five-fold dilution).

Viral Effects on Different Size Groups

Different size groups of naive snakeheads consisting of fingerlings, 0.93 - 1.21 g and fry, 0.12 - 0.21 g were exposed to virus isolate 95-96. Three replicates of 10 fingerlings per aquarium were injected intramuscularly (10^5 $TCID_{50}$ per fish) and a similar number of replicates of 10 fry were immersed in viral suspension (10^5 $TCID_{50}$ per

ml) for 1 h then diluted to 5 liters (a five-fold dilution) at water rearing temperatures of 21 - 22 °C. Each size group had three replicates of controls challenged only with MEM-4.

Monitoring of Experimental Fish

All test aquaria were checked daily for fish mortality and for the development of EUS-like lesions according to the criteria in Table 2.3.

Tissue Sampling for Virus Recovery

Experimental fish were sampled, anaesthetized in 10 mg per L MS222, and then disinfected externally with a cotton swab soaked in 70 % ethanol before dissection. Primary isolations of the virus were made from pooled tissue filtrates excised from lesions/muscle, liver, gonad, kidney, spleen and gills of the experimentally infected naive snakeheads.

Twenty-four hours confluent CCO cells in 24-well plates were used for detection of virus in sampled tissue filtrates. CPE was consistently detected over several passages with virus positive filtrates. Blind passages were carried out on negative samples at least three times. GIBCO media, reagents and antibiotics used were prepared as described in Chapter 7.

Statistical Tests

Data on the infection experiments with fry and fingerlings were subjected to arcsine transformation then tested for significance using the Student's *t* test.

RESULTS

Naive snakeheads with the three viral isolates (95-1, 95-4 and 95-96) caused the initiation of very slight dermal lesions (+/-) at the injection site as early as d 3 to 5 following intramuscular injection (Table 9.1). Development of slight lesions (+) from 3 d to 7 d post-treatment occurred. The lesions then progressed to ++ by 6 - 12 d and subsequently to moderate lesions (+++) by d 10 - 12 (Fig. 9.1). Except for one fish that died before manifesting any lesion, all virus-injected fish developed dermal lesions. However, the lesions did not progress to deep ulcers (++++) commonly exhibited by severely EUS-affected snakeheads. Overall mean mortalities were higher in the virus-injected fish (72 %) compared to the mean mortalities of sham-injected fish (33 %).

Among virus-injected naive fish, only the test fish injected intramuscularly with the virus and reared at the 20 - 22.5 °C manifested dermal lesions at the site of injection (Table 9.2). IP virus-injected fish died without expressing any signs of disease. Fish injected either IM or IP with the 95-1 virus at ambient water temperature (28 - 32 °C), however, did not manifest any lesions. In addition, the virus was reisolated from the test fish in the 20 - 22.5 °C treatment but not with the test fish in the 28 - 32 °C treatment. Deaths were observed only among the IM and IP virus-injected test fish maintained at low rearing water temperatures (20 - 22.5 °C).

In the experiment using unpropagated viral isolate and virus passaged three or four times, the results showed that both types of viral suspension can induce dermal lesions (+ to ++) at the injection site if the virus concentration is > 10^3 $TCID_{50}$ per fish (Table 9.3). Almost all virus-treated fish died during the course of the experiment. Most virus-injected fish died before lesions advanced to the +++ stage. The control fish did not

Table 9.1. Lesion and mortality development at 20 - 22.5 °C in naïve juvenile snakeheads (*Ophicephalus striatus*) injected intramuscularly (IM) with the virus isolates 95-1 (10^6 $TCID_{50}$ per fish), 95-4 (10^5 $TCID_{50}$ per fish) and 95-96 (10^6 $TCID_{50}$ per fish).

Viral Isolate	$TCID_{50}$ Per Fish	Replicate	Fish No.	Day Post-exposure 3	4	5	6	7	8	9	10	11	12
95-1	10^6	1	1	-*	+/-	+/-	+/-	+/- d					
			2	-	-	+/-	+/-	+	+	++	++	++	+++
		2	1	+/-	+/-	+/-	+/-	+/- d					
			2	-	-	- d							
		3	1	-	+/-	+/-	+/-	+/- d					
			2	-	-	+/-	+/-	+	+	+	++	++ d	
95-4	10^5	1	1	-	+	+	+	+	+	+	+	+	++
			2	-	+/-	+/-	++	++	++ d				
		2	1	-	+/-	+/-	+/-	+/- d					
			2	-	-	+/-	+/-	+	+ d				
		3	1	-	+/-	+/-	+	+ d					
			2	-	-	+/-	+	+ d					
95-96	10^6	1	1	+	+	+	+	+	+	++	+++	+++	+++
			2	+/-	+/-	+/-	+/-	+/-	+	++	++	++	++
		2	1	-	+/- d								
			2	-	-	+	+	+	+	+	++	+++d	
		3	1	-	-	+	+ d						
			2	-	-	+/-	+/-	+/-	+	+	+	+	++
Control		1	1	-	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-	-
		2	1	-	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	- d			
		3	1	-	-	-	-	-	-	-	-	-	-
			2	-	-	-	- d						

* see Table 2.3 for lesion grade criteria

d dead

Fig. 9.1. Juvenile snakeheads (*Ophicephalus striatus*) at 20 - 22.5 °C following intramuscular injection with sterile medium (a) and with 10^6 $TCID_{50}$ per fish of the 95-96 virus isolate showing + (c) and +++ (b and d) lesions.

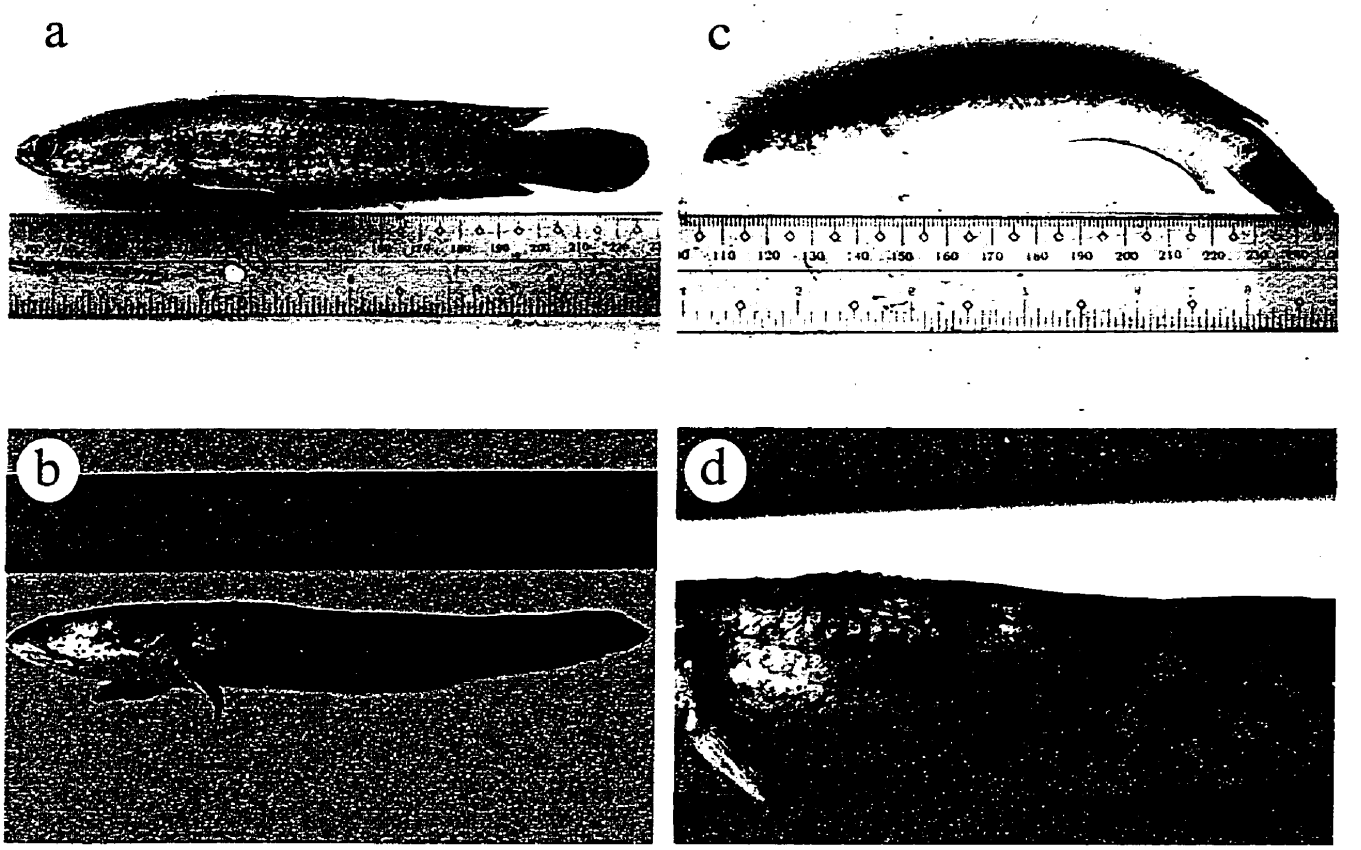

a
c
b
d

Table 9. 2. Effect of temperature on lesion development and deaths of the 95-1 virus isolate following intramuscular (IM) and intraperitoneal injections (IP) (10^6 $TCID_{50}$ per fish) in naive snakehead (*Ophicephalus striatus*) juveniles.

Viral Isolate	Water Temp.(°C)	Treatment	Fish #	Days Post-exposure 4	5	6	7	8	9	10	11	12
95 - 1	20 - 22.5	IM	1	-*	+	+	+d					
			2	-	+	+	+	+	+	+	+	++
		IP	1	-	-d							
			2	-	-	-	-	-	-	-d		
	28 - 32	IM	1	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-
		IP	1	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-
Control	20 - 22.5	IM	1	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-
		IP	1	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-
	28 - 32	IM	1	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-
		IP	1	-	-	-	-	-	-	-	-	-
			2	-	-	-	-	-	-	-	-	-

d dead

* see Table 2. 3 for lesion grade criteria

Table 9.3. Effects of different unpropagated viral isolates (P_o) and virus passaged 3 or 4 times ($P_{3\ or\ 4}$), on lesion development and mortality of naive snakehead (*Ophicephalus striatus*) juveniles at 20 - 22.5 °C following intramuscular injection (IM).

Viral Isolate	$TCID_{50}$ Per Fish	Fish #	Days Post-exposure 3	4	5	6	7	8	9	10	11	12	13	14
95-1 P_0	10^2	1	d*											
		2	-	-	-	-	- d							
95-1 P_3	10^7	1	-	-	+	+	d							
		2	-	-	+	+	+	+	+	+	+	++	++d	
95-4 P_0	10^5	1	-	-	+	+	+	+	+	+ d				
		2	-	-	+	+	+	+	+	+	++	++	++d	
95-4 P_4	10^6	1	-	+/-	+	+	+	+	+	+	+	++	++	++d
		2	-	-	+/-	+/-	++	++ d						
95-5 P_0	10^4	1	-	-	d									
		2	-	-	-	-	-	-	-	-	-	-	-	-
95-5 P_3	10^8	1	d											
		2	-	-	+	+ d								
Control	0	1	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-

P passage number in tissue culture
* see Table 2.3 for lesion grade criteria
TCID tissue culture infectious dose
d dead

die.

Intraperitoneal injections of the virus again did not induce lesions in test fish (Table 9.4). However, mortalities occurred among the majority (75 %) of virus-injected fish and none in control fish.

A viral dose of at least 10^3 $TCID_{50}$ per fish was required to induce EUS-like lesions at the IM injection site in naive snakeheads (Table 9.5). Onset of lesions was detected in 5 d. The most advanced lesion detected was +++. Mortalities appeared to be directly related to viral dose as the highest dose resulted in total mortality by d 10 whereas fish injected with 10^2 to 10^5 $TCID_{50}$ per fish yielded 80 % mortalities on d 10 - 15. No deaths were observed among sham-injected fish.

All viral isolates from the different organ sources induced dermal lesions at the site of IM injection (Table 9.6). Onset of lesions occurred between d 3 - 9 while advanced lesions (+++) were observed at d 10 - 12 on fish from which virus was isolated from livers, gills and lesion material. Mortality occurrences were comparable with those of control fish.

Naive fish exposed to the 95-96 virus by different routes showed that only the IM and SD test fish developed lesions resulting in mortalities of 4 of 5 and 2 of 5 test fish, respectively (Table 9.7). Dermal lesions initially appeared on d 5 post-injection graduating to moderate lesions by d 9 - 11. One IM fish even developed an ulcerative lesion by d 12. When injected IP virus did not induce lesions but caused 80 % mortality. Virus given orally by intubation and by bath did not induce lesions or deaths. Control fish did not manifest any pathologic signs.

Table 9.4. Lesion development and deaths at 20 - 22.5 °C among naive juvenile snakeheads (*Ophicephalus striatus*) injected intraperitoneally (IP) with different unpropagated (P_o) or passaged (P_3) virus isolates.

Viral Isolate	$TCID_{50}$ Per Fish	Fish #	Days Post-exposure 3	4	5	6	7	8	9	10	11	12
95-1 P_0	10^2	1	-*	-	d							
		2	-	d								
95-1 P_3	10^7	1	-	-	d							
		2	-	-	-	-	-	-	-d			
95-4 P_0	10^5	1	d									
		2	-	-	-	-	-	-	-d			
95-4 P_3	10^6	1	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-
95-5 P_0	10^4	1	d									
		2	-	-	-	-	-	-	-	-	-	-
95-5 P_3	10^8	1	-	-	d							
		2	-	-	-	-	d					
Control	0	1	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-

P passage number in tissue culture
* see Table 2.3 for lesion grade criteria
TCID tissue culture infectious dose
d dead

Table 9.5. Dose response at 20 - 22.5 °C (lesion development and deaths) of naive snakehead (*Ophicephalus striatus)* juveniles following intramuscular injection with different concentrations of the 95-96 virus isolate.

Dose Per Fish ($TCID_{50}$)	Days Post-exposure 3	5	7	10	12	15
10^7	4^n-*1d	2+/++2d	1+1d	1d		
10^6	4-1d	4+/++1d	2+/++1d	2+/++	1+1d	1+++
10^5	5 -	4+1d	2+2d	1++1d	1+++	1+++
10^4	5 -	1-2+2d	2+1d	2+	2+	1+1d
10^3	5 -	3+/++2d	3+/+++	3+/+++	3+/+++	1++2d
10^2	5 -	5 -	5 -	4-1d	3-1+	2-2d
Control	5 -	5 -	5 -	5 -	5 -	5 -

TCID tissue culture infectious dose
* see table 2.3 for lesion grade criteria
n number of fish
d dead

Table 9.6. Lesion development and deaths at 19 - 23 °C in naive juvenile snakeheads (*Ophicephalus striatus*) injected (IM) with various virus isolates (10^6 $TCID_{50}$ per fish) isolated from different organ filtrates of epizootic ulcerative syndrome (EUS)-lesioned fish and propagated in channel catfish ovary (CCO) cells.

Viral Isolate	Organ Source	Fish #	Days Post-exposure 2	3	4	5	6	7	8	9	10	11	12
95-2	Liver	1	d										
		2	-*	-	+/-	+/-	+/-	+/-	+	+	++	+++	+++
95-4	Kidney	1	-	-	+/-	+/-	+/-	+/-	+/-	+ d			
		2	-	-	-	-	-	-	-	+/-	+	++	++
95-15	Spleen	1	-	d									
		2	-	-	-	+/-	+/-	+/-	+/-	+	+	+	+
95-91	Gonad	1	-	-	+/- d								
		2	-	-	-	+/-	+/-	+/-	+/-	+/-	+	+	+
95-117	Gills	1	-	-	- d								
		2	-	+/-	+/-	+/-	+/-	+/-	+	++	+++	+++	+++
95-96	Lesion	1	-	+/-	+	+	+	+	+	++	+++	+++	+++
		2	-	+/-	+/-	+/-	+/-	+/-	+	++	++	++	++
Control		1	-	-	-	- d							
		2	-	-	-	-	-	-	-	-	-	-	-

* see Table 2.3 for lesion grade criteria
d dead

Table 9.7. Lesion development and deaths at 19 - 23 °C of naive juvenile snakeheads (*Ophicephalus striatus*) following intramuscular (IM), intraperitoneal (IP), intubated and subdermal (SD) treatments with the 95-96 EUS virus at a dose of 10^6 'TCID$_{50}$ per fish.

Portal of Entry	Days Post-exposure 5	7	10	12	15
IM	3^n+/++*2d	3+/++	2+/+++1d	2+/++++	1++++1d
Control	5 -	5 -	5 -	5 -	5 -
IP	5 -	5 -	4-1d-	2-2d-	1-1d-
Control	5 -	5 -	5 -	5 -	5 -
Intubation	5 -	5 -	5 -	5 -	5 -
Control	5 -	5 -	5 -	5 -	5 -
SD	2-3+	3+2d	1-2+/+++	1-2+++	1-2+++
Control	5 -	5 -	5 -	5 -	5 -
Bath	5 -	5 -	5 -	5 -	5 -
Control	5 -	5 -	5 -	5 -	5 -

n number of fish
* see Table 2.3 for lesion grade criteria

Virus-bathed fry and fingerlings died without showing signs of disease. Virus-exposed fry began to die by d 2 post-exposure with all fish dead by d 4 - 5 (Table 9.8). Mortalities of viral-exposed fingerlings, first occurred on d 5 (mean = 17 %) and gradually increased to 100 % in all three replicates by d 9. Mortalities between the two virally-exposed fish size groups were significantly different ($P<0.01$) on d 4 and 5 (fingerlings took longer to die than fry). Inoculated virus was recovered from the lesions and viscera of fish infected via IM and IP routes with the virus for at least 12 d post-exposure. Control fish were negative for the virus.

DISCUSSION

In general, the present study demonstrated that the '95 series virus isolates can cause dermal pathology among juvenile snakeheads when injected IM. The induced infection site lesions ranged from very slight lesion (+/-) by d 3, slight lesion (+) by d 5, to moderate lesions (+++) by d 10. Clinical signs of EUS consisting of slight lesions or small red spot development was reported earlier as being correlated with the early stages of the disease (Cruz-Lacierda 1995; Menasveta 1985; Roberts *et al.* 1986; Shresta 1990). The onset of mild (+) and moderate lesions (+++) among artificially infected fish occurred in 5 and 10 - 12 d, respectively, which was comparable to the results with naive snakeheads naturally exposed to the EUS-associated virus via diseased or apparently healthy fish carriers. These EUS signs developed in 6 and 9 - 11 d, respectively, (Chapter 8; Balasuriya *et al.* 1990).

Table 9.8. Cumulative deaths among naive snakehead (*Ophicephalus striatus*) fry (n=20) and fingerlings (n=10) exposed to the 95-96 EUS virus by bath (10^5 $TCID_{50}$ per ml for 1 h) at 20 - 22 °C.

Treatment	Replicate No.	Days Post-exposure 1	2	3	4	5	6	7	8	9	10
Fry											
Test	1	0	0	12	20	20					
	2	0	0	2	20	20					
	3	0	2	12	18	20					
	mean	0	0.7	8.7	19	20					
Control	1	0	0	0	0	0					
	2	0	0	0	0	0					
	3	0	0	0	0	0					
	mean	0	0	0	0	0					
Fingerlings											
Test	1	0	0	0	0	1	1	2	6	10	
	2	0	0	0	0	4	4	5	7	10	
	3	0	0	0	0	0	3	8	9	10	
	mean	0	0	0	0	1.7	2.7	5	7.3	10	
Control	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	2	2	2	2
	3	0	0	0	0	0	0	0	0	0	0
	mean	0	0	0	0	0	0	0.7	0.7	0.7	0.7

In all of the experiments, a total of 85 juvenile snakeheads were injected intramuscularly with the '95 series virus isolates. Of these, 73 fish (86 %) developed lesions. Those that did not manifest clinical signs either died before the expected onset of lesions or were given sublethal (too small) doses. A notable observation was that, except for one fish, the dermal lesions did not advance to deep ulcers such as those observed under natural conditions (Chapter 8). Lesion development was restricted only to the site of the injection, even in viremic conditions. Similar observations have been made among largemouth bass injected IP with an iridovirus which showed inflamed lesion at the site of injection only (Plumb *et al.* 1996).

The predominant EUS lesion among snakeheads is often an extensive and ulcerative lesion that penetrates deeply into the muscle. Such extensive lesion was not observed among naive fish, which were experimentally injected with the viruses. This may be because the viruses initiate only a primary dermal lesion (+) as has been suggested earlier (Roberts *et al.* 1986; Menasveta 1985).

It is hypothesized that the lesion progresses into more extensive and advanced ulcers upon secondary infection with the bacterium most prevalent in the fish environment, *A. hydrophila* (Chapter 2; Llobrera and Gacutan 1987; Torres *et al.* 1990) and/or with fungal pathogens, such as *Achlya* spp. or *Aphanomyces* spp. (Callinan *et al.* 1995a; Lilley and Roberts 1997; Willoughby *et al.* 1995). Bacterial and fungal pathogens isolated from EUS lesioned fish in the Philippines and Thailand can experimentally induce dermal lesions (Chapters 2 and 4; Callinan *et al.* 1995b; Torres *et al.* 1990). In addition, snakeheads are bottom dwelling fish and the *A. hydrophila* population is greater in sediments than in overlying waters. This may readily cause more secondary infections

among the lesioned benthic fish (Chapter 2). Hence, despite extensive and advanced lesions commonly observed among EUS-affected snakeheads, these appear to be more resilient and may suffer considerable clinical tissue destruction over a period of weeks before they succumb (Roberts *et al.* 1994).

It is apparent from this study that IM or SD exposures to the '95 series virus isolates were more effective routes for inducing dermal signs in naive juvenile snakeheads. Snakeheads being cannibalistic in nature, have a tendency to bite other fishes, which may provide the opportunity to introduce the virus into a naive fish. Hence, the route of infection of juvenile snakeheads with virus is likely through the injured skin or musculature.

Nguyen *et al.* (1996) suggested that results of studies related to the entry and primary replication sites of virus in host fish partly depends on the mode of entry employed. Their studies with striped jack nervous necrosis virus (SJNNV) showed that although the virus apparently multiplied in the spinal cord, of both naturally and experimentally virus-immersed 2-d old larvae, its entry was most likely through the skin epithelium. They further suggested that the epithelial cells might be susceptible to the virus infection only in the very early developmental stage of the host fish. This may also be the case with the EUS virus infection of snakehead fry and fingerlings in this study. Furthermore, in immunohistological studies with infectious hematopoietic necrosis virus (IHNV), Yamamoto *et al.* (1990) found that the epithelium of the skin was a major site of IHNV replication and was more important as the entry site than were the gills. In studies on the entry and progression of IHNV in steelhead trout (*O. mykiss*) fry, Drolet *et al.* (1994) showed that the epithelial cells of the skin and of virtually every organ were virus-

positive during the pre-epizootic period and became virus-negative during the epizootic period of the experimental infection. This finding suggested that IHNV infection of the epithelial cells was transient and not highly productive as in the hematopoietic tissue of the kidney.

Among naive juveniles, IP, IM and SD injections were responsible for mean mortalities of 86, 72 and 40 %, respectively (Table 9.7). Although IP injection of the viruses failed to produce dermal pathology, the significant mortalities attributed to viral injection are meaningful. The IP injection of the virus likely facilitated entry into the circulatory system which may have resulted in its rapid dissemination to the different susceptible organs of naive fish. It apparently multiplied in the test juvenile fish, causing death without causing external clinical signs. That the virus did indeed propagate in test fish was shown by the recovery of the virus from the visceral organs of the test fish in this study. Also, virus isolation was achieved from muscle, livers, gonads, spleens, kidneys and gills of naive snakeheads after natural EUS disease transmission experiments conducted in Chapter 8.

The viral dose for successful inducement of dermal lesions on juvenile snakeheads was at least 10^3 $TCID_{50}$ per fish via intramuscular injection. In studies with two-year old kokanee, *O. nerka*, Traxler (1986) showed that fish undergoing losses due to IHN usually have viral titers in excess of 10^4 plaque forming units (PFU) per g of tissue.

The effect of the 95-96 virus isolate on snakeheads is influenced by the age of the fish. Fry (approximately less than 2 weeks old) and fingerlings (approximately 2 - 6 weeks old) exposed to viral dose of 10^5 $TCID_{50}$ per fish in this study manifested significant mortalities 4 and 6 d post viral exposure, respectively (Table 9.8). Apparently,

the younger the fish, the more rapid the death as snakehead juveniles (approximately > 6 weeks – 3 months old) given a similar treatment were unaffected (Table 9.7). Thus, snakehead fry are more susceptible to the virus than fingerlings. Similarly, Traxler and Rankin (1989) observed that mortalities of sockeye fry could occur when viral titers are as low as 10^4 PFU per g of whole fish. Juvenile snakeheads develop dermal lesions at 5 d following IM injection and some mortalities after IM and IP injections of the virus in this study. Such variations in responses to viral pathogen exposure among different size groups of fish have been reported for other viral infections. Yamamoto *et al.* (1990) in their experiments with IHN concluded that the virus enters and multiplies rapidly in target organs of very young rainbow trout fingerlings, but in older, somewhat more resistant fish, the virus appeared to enter, multiply and persist as lesions on the skin for a period of time before viremia and general spread of the infection. This decrease of sensitivity to viral infection with increasing age was similarly noted in viral nervous necrosis (VNN) virus in *Lates calcarifer* (Munday *et al.* 1992) and among rainbow trout with infectious pancreatic necrosis (IPN) virus (Dorson and Torchy 1981). Thus, young fish with viral infection often do not live to maturity although some are able to overcome the infection by developing partial or complete immunity (Chapter 6).

This is the first report on the successful transmission of tissue-cultured viruses from EUS-affected fish to snakeheads. Frerichs *et al.* (1993), using viral isolates from EUS-lesioned fish from Thailand, failed to induce dermal lesions among injected fish. Frerichs *et al.* (1993) used IP injections and bath exposures with 3-month-old striped snakeheads.

Our previous experiments using IM injections of the 91-97 virus isolate failed to induce dermal lesions (Chapter 7). This may be attributed to either attenuation of the virus or to other factors as explained in Chapter 11. Attenuation from numerous passages of the virus in cell culture was implicated as the cause of loss of virulence of pike fry rhabdovirus to pike, *Esox lucius* (Haenen and Davidse 1993). In subsequent studies in Thailand, Kanchanakhan (1996), reported on mortalities among snakehead fry and skin damage in juveniles artificially exposed to new isolates of rhabdovirus from EUS fish.

The present experiments show that temperature plays a very important role in the pathogenesis of EUS. Lesion development and mortalities occurred only among virus-injected snakeheads reared at the lower temperature of 20 - 22.5 °C and not among those reared at 28 - 32 °C. This lower temperature range corresponds with that of the water temperature during cooler months of December through February when natural outbreaks of EUS among freshwater finfishes occur in the Philippines and other EUS-affected countries. In addition, data in Chapter 7 show that the higher water temperature of 28 - 32 °C decreases the survival of ERV. The influence of temperature upon pathogenesis of viruses is a complex interaction between the virulence of the virus and the defense mechanism of the host. Each likely plays a role in whether or not EUS occurs.

In conclusion, these results show that juvenile snakeheads with no apparent prior exposure to the virus can be susceptible to both infection and subsequent mortality. Temperature has a pronounced effect on the pathogenicity of EUS-associated viruses. IM injection of the virus causes lesions and then mortalities while IP injection causes mortalities only at the lower water temperatures. All virus isolates had approximately similar virulence. Increased passages of the virus in tissue culture and the organ source of

the virus isolate had little effect on virulence. As low as 10^3 $TCID_{50}$ dose per fish could cause lesions and mortalities.

CHAPTER 10

VIRULENCE AND ANTIGENICITY OF THE EPIZOOTIC ULCERATIVE SYNDROME (EUS) RHABDOVIRUS AND THE OCCURRENCE OF DEFECTIVE INTERFERING VIRAL PARTICLES IN TISSUE CULTURES

INTRODUCTION

A number of researchers have isolated rhabdoviruses from snakeheads that displayed signs of EUS in Thailand, Myanmar and Sri Lanka (Frerichs *et al.* 1986; Kasornchandra *et al.* 1992; Lilley and Frerichs 1994). Upon subsequent testing for pathogenicity, however, one isolate was reported to be pathogenic whereas others were not (Kanchanakhan 1996). Frerichs *et al.* (1989a) reported the isolation of six EUS rhabdoviruses from Thailand and Myanmar, which upon infection of healthy snakeheads, did not cause signs usually associated with EUS. Kanchanakhan (1996) reported isolation of nine viruses from EUS fish in Thailand; one of which caused mortalities among experimentally challenged snakehead fry and dermal EUS-like lesions in snakehead juveniles.

In previous studies, several viral isolates were recovered from snakeheads which displayed signs of EUS in the Philippines, two of which were extensively used in this

study: #91-97 virus isolate (I) which was non-pathogenic to naive snakeheads (Chapters 6 and 7) and #95-96 virus isolate (III) which was pathogenic (Chapter 8 and 9). This portion of my study was initiated to identify the difference between these isolates as regards their pathogenicity and antigenic relatedness.

MATERIALS AND METHODS

Virus Isolates

Virus isolates #91-97 (I) and #95-96 (III), which were previously isolated from EUS-affected snakeheads, were used. Virus I when subcultured in CCO cells was designated virus II while subcultures of virus III in SHS cells were coded virus IV. In addition, another viral isolate #96-23 (V and VI), which was isolated from EUS-affected snakeheads in January 1996, was studied (Table 10.1). All virus isolates were propagated separately and aliquots preserved at -70 °C.

Cell Cultures

The following cell lines were used: AS, BF2, CFS, CCO, CHSE, EPC, RTG-2, and SHS. The procedure for culture, incubation, media, reagents and antibiotics used are described in Chapter 7.

Table 10.1. Viruses isolated from EUS-affected snakeheads (*Ophicephalus striatus*) in the Philippines.

Isolate #	Code	Cells For Primary Isolation	Cells For Continuous Culture	Date Isolated (January)
91-97	I	SHS	SHS	1991
91-97	II	SHS	CCO	1991
95-96	III	CCO	CCO	1995
95-96	IV	CCO	SHS	1995
96-23	V	SHS	SHS	1996
96-23	VI	CCO	CCO	1996

CCO Channel catfish ovary cells
SHS Snakehead spleen cells

Virus Replication in Different Cell Lines

Ten-fold serial dilutions of viral isolate I, were inoculated onto the following cells: AS, BF2, CHS, CCO, CHSE, EPC, RTG2 and SHS in 96-well tissue culture plates as described in Chapter 7. Cytotoxicity was checked daily for 7 d.

Virus Replication at Different Incubation Temperatures

Confluent SHS cells in 25 cm^2 tissue culture flasks (Falcon) were inoculated with 1.5 ml of a 10^3 $TCID_{50}$ per ml suspension of the virus isolate I as described in Chapter 7. Seven inoculated flasks were incubated at each of the following temperatures: 15, 20, 25, 30 and 37 °C. Each day for 7 d, one flask incubated at each test temperature was removed and the contents frozen at –70 °C. These flasks were rapidly thawed and the virus titer of each flask was determined using 96-well plates as described in Chapter 7.

Virus Assays

CCO and SHS cells were grown to confluency in 96-well tissue culture plates in either L15-4 or MEM-4 with HEPES before inoculation with the test viruses. Viral titers were determined by the end-point dilution assay ($TCID_{50}$) (Rovozzo and Burke 1973) after 5 d incubation at 20 °C.

Growth of virus isolates in CCO and in SHS cells was also studied. Viral isolates III, IV, V and VI were passed undiluted and diluted (10^{-3}), weekly in their respective cells. The cells were observed for cytotoxicity in each passage until the 36th passage.

Experimental Fish

Wild, healthy snakehead (*O. striatus*) juveniles, 17 - 40 g, were collected from several rivers in Iloilo, Philippines. Since EUS has never been observed in Iloilo and the virus has not been isolated from sampled Iloilo snakeheads, these fish were considered naive. The fish were acclimated for 1 week in freshwater at ambient temperature (28 - 32 °C) and for at least 3 d at 20 - 22.5 °C before initiating infection experiments. When used for experimental purposes, the fish were placed in 5-liter aquaria containing cooled, pre-boiled or UV-sterilized unchlorinated freshwater. One fish was placed in each aquarium.

Infection Experiments

Naive snakehead juveniles were injected intramuscularly (IM) with 0.1 ml of viral isolates II or I.

In the second experiment, virus isolate V, passaged undiluted or diluted (10^{-3}) 11 times (P11) in SHS cells and isolate VI propagated undiluted in CCO cells, were injected IM into snakehead juveniles. Two fish were tested for each treatment in 20 - 22.5 °C rearing water temperature.

In the third experiment, viruses III and IV undiluted and diluted (10^{-3}) in SHS cells were injected IM into naive snakehead juveniles. Five fish were tested for each treatment in 20 - 22.5 °C rearing water temperature.

In all experiments, sterile MEM-4 was used to inject the controls, and the viral dose of the inoculated isolates was 10^6 $TCID_{50}$ per fish. All fish were checked daily for death and development of lesions according to the criteria set out in Table 2.3.

Virus Purification

Viral isolates I, II and III were propagated in their respective cells, in media supplemented with 2 % bovine serum albumin (BSA) (Sigma) in lieu of FBS and then purified following the method described in Chapter 7. The final viral pellets were resuspended in Tris buffer and stored at -70 °C.

Electron Microscopy

Purified virus III and I were negatively stained with ammonium molybdate and suspended in formvar-carbon-coated copper grids for viewing under the transmission electron microscope (Philips EM300).

Production of Antibody

Antiserum against purified virus isolates I, II and III were produced by injection of individual New Zealand white female rabbits. For injection, the purified virus was emulsified in incomplete Freund's Adjuvant (IFA). Approximately 105 - 112 ug of purified viral proteins were injected subcutaneously and the rabbits were allowed to rest for 2 weeks after which they were boosted with 50 - 90 ug of the antigens. Blood was collected from the marginal ear vein, allowed to clot at 37 °C for 1 h and then incubated overnight at 4 °C. The serum was collected by centrifugation and kept at -20 °C until used. Normal rabbit serum that acted as a negative control was collected before immunization.

Protein Quantification

Protein concentration of the purified virus was estimated using the Bio-Rad Protein Assay kit.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis of the purified virus isolates I, II and III was carried out according to the method of Laemmli (1970), using a 4 % stacking gel and a 12 % separating gel 1 mm thick. Separation of viral proteins under denaturing conditions with 2-mercaptoethanol was done using the Bio-Rad mini-Protean II system. Low molecular weight protein standards (Bio-Rad) were included as markers in each run. Following electrophoresis, gels were silver-stained as described in Johnstone and Thorpe (1987). All chemicals used in electrophoresis were purchased from Sigma.

Western Blotting

Discontinuous SDS-PAGE of samples was performed as described above. However, prestained markers of low molecular mass (Bio-Rad) were included in the gel. The separated polypeptides were then transferred electrophoretically onto 0.45 um nitrocellulose (N/C) membrane (Bio-Rad), using a semi-dry transblot apparatus (2117-250 Novablot LKB, Bromma, Sweden) at 0.8 mA/cm^2 for 1-2 h. All subsequent manipulations were performed at room temperature. The N/C membranes were washed briefly in Tris-buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5), and then blocked for 1 h with 3 % solution (w/v) of gelatin in TBS. The N/C membranes were then washed twice in Tween 20-containing TBS (TTBS) and exposed to a solution of rabbit

anti-I, anti-II or anti-III sera (diluted 1:200 in 1 % gelatin - TTBS-antibody buffer) overnight with gentle shaking. The N/C membranes were washed twice in TTBS and then exposed to a solution of horseradish peroxidase (HRP) conjugated to goat anti-rabbit IgG (Bio-Rad), diluted 1:1500 in antibody buffer for 1 h with gentle shaking. The solution was then discarded and the N/C membranes were washed twice in TTBS and once in TBS. The N/C membranes were developed by addition of the HRP substrate (4-chloro-a-naphthol, Bio-Rad). Developed blots were kept in the dark until photographed.

RESULTS

High dilutions of virus isolate I caused CPE in BF2, CCO, CFS and SHS cells (Table 10.2). However, when the virus inocula were undiluted or used at low dilutions. CPE developed only in CCO and CFS cells.

In SHS cells, the absence of CPE was initially detected among wells inoculated with the undiluted virus I at d 2, 2 and 1 at temperatures of 20, 25 and 30 °C, respectively (Table 10.3). At 15 and 37 °C, undiluted virus I caused CPE in d 1 to 4 and 5, respectively. With the 10-fold diluted viral I inoculum, no CPE was observed at incubation temperatures of 25 and 30 °C, by d 3 post-inoculation and at 20 °C by d 4. At 25 °C, no CPE was apparent with SHS cells 6 d after inoculation with the 10^{-2} diluted virus I.

Virus isolates IV and V, when passed undiluted and diluted (10^{-3}) in SHS cells until the 36th cell passage, showed no CPE with the undiluted inoculum in most passages. It was noted, however, that at every third to fifth passage, the virus would

Table 10.2. Replication of different dilutions of the 91-97 virus isolate in different cell lines.

Virus Dilution	Cell Line AS	BF2	CCO	CFS	CHSE	EPC	RTG-2	SHS
0	-	-	+	+	-	-	-	-
10^{-1}	-	-	+	+	-	-	-	-
10^{-2}	-	+	+	+	-	-	-	+
10^{-3}	-	+	+	+	+	-	-	+
10^{-4}	-	+	+	+	-	-	-	+
10^{-5}	-	+	+	+	-	-	-	-
10^{-6}	-	-	-	-	-	-	-	-

AS Atlantic salmon cells
BF2 Bluegill fry cells
CCO Channel catfish ovary cells
CFS Catfish spleen cells
CHSE Chinook salmon epithelial cells
EPC Epithelioma papulosum cyprini cells
RTG-2 Rainbow trout gonad cells
SHS Snakehead spleen cells
\- no cytopathic effect detected
\+ cytopathic effects detected

Table 10.3. Replication of different dilutions of the 91-97 virus isolate (I) in snakehead spleen cells from an initial titer of 10^6 $TCID_{50}$ per ml at different incubation temperatures.

Temperature	Virus	Day Post-exposure						
(°C)	Dilution	1	2	3	4	5	6	7
15	10^{-0}	+	+	+	+	-	-	-
	10^{-1}	+	+	+	+	+	+	+
	10^{-2}	-	+	+	+	+	+	+
	10^{-3}	-	+	+	+	+	+	+
	10^{-4}	-	+	+	+	+	+	+
	10^{-5}	-	+	+	+	+	+	+
	10^{-6}	-	-	-	-	-	-	+
20	10^{-0}	+	-	-	-	-	-	-
	10^{-1}	+	+	+	-	-	-	-
	10^{-2}	+	+	+	+	+	+	+
	10^{-3}	+	+	+	+	+	+	+
	10^{-4}	-	+	+	+	+	+	-
	10^{-5}	-	-	-	+	+	-	-
	10^{-6}	-	-	-	-	-	-	-
25	10^{-0}	+	-	-	-	-	-	-
	10^{-1}	+	+	-	-	-	-	-
	10^{-2}	+	+	+	+	+	-	-
	10^{-3}	+	+	+	+	+	+	+
	10^{-4}	+	+	+	+	+	-	-
	10^{-5}	-	-	-	-	-	-	-
30	10^{-0}	-	-	-	-	-	-	-
	10^{-1}	+	+	-	-	-	-	-
	10^{-2}	+	+	+	+	+	+	+
	10^{-3}	+	+	+	+	+	+	+
	10^{-4}	+	+	-	+	-	+	+
	10^{-5}	-	+	-	-	-	-	-

cause CPE. On subsequent passages thereafter, the virus would again not cause CPE in the first and second passages. Cells inoculated with the diluted virus, however, consistently exhibited CPE in every passage, 3 - 5 d post-inoculation.

When isolates II and I were injected IM into naive fish, no lesions developed in any test fish. Undiluted viral isolates III and IV were virulent to the naive snakehead juveniles, inducing dermal lesions (Table 10.4). When naive snakeheads were injected IM with isolates V and VI, the undiluted inocula were not pathogenic while the serially diluted inocula induced dermal lesions (Table 10.5).

Figure 10.1 shows the transmission electron micrographs of the purified I and III viral isolates. Both show bullet-like morphologies typical of rhabdoviruses.

Figure 10.2 shows the SDS-PAGE protein silver-stained bands of isolates I, II and III. The EUS rhabdovirus protein bands corresponded to the L, G, N and M1, M2 viral proteins. In addition, the three viral isolates had similar protein bands except for isolate III, which exhibited an additional protein band with a molecular weight of approximately 29 kD.

Immunoblotting of these protein bands with their polyclonal anti-sera revealed the specific binding of each antiserum with its specific virus (Fig. 10.3). In addition, anti-I serum showed some common bands with II and III. Anti-II serum had bands common with those of I or II. Recognition of the protein bands of virus I by anti-III serum was less apparent but showed some common antigens with II as well.

Table 10.4. Lesion development at 19 - 23 °C in naive snakeheads (*Ophicephalus striatus*) injected intramuscularly with the virus isolate 96-23 P_{11}(10^6 $TCID_{50}$ per fish) propagated in snakehead spleen cells (SHS) and in channel catfish ovary cells (CCO) using undiluted and diluted (10^3) inocula.

Treatment	Virus Code	Day Post-exposure 3	5	7	10	12	15	20
10^0 SHS	V	2^n-*	2-	2-	2-	2-	2-	2-
10^3 SHS	V	2+	2+	2+	2+	1+1++	1-1+	1-1+
10^0 CCO	VI	1-1+	1-1+	1-1+	2-	2-	2-	2-
Control		2-	2-	2-	2-	2-	2-	2-

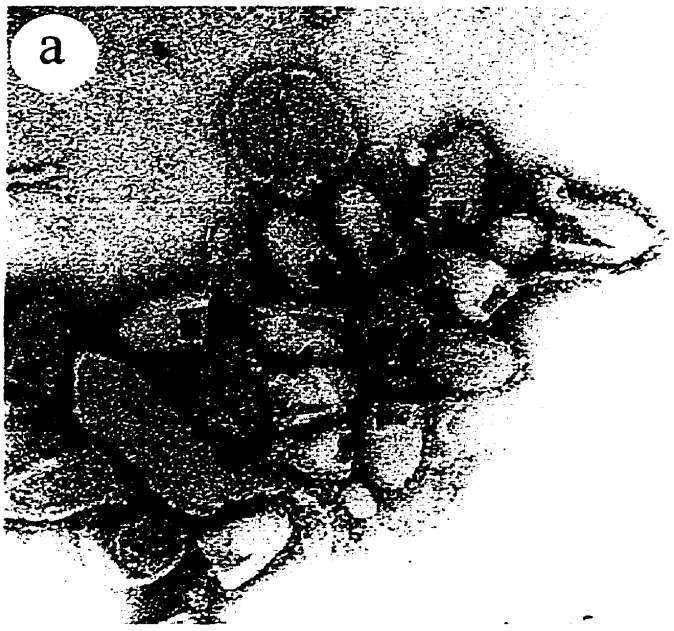
n number of test fish
* see Table 2.3 for lesion grade criteria
number of fish per treatment = 2

Table 10.5. Lesion development at 19 - 23 °C in naive snakeheads (*Ophicephalus striatus*) injected intramuscularly with 10^6 $TCID_{50}$ per fish of the virus isolate 95-96 propagated in snakehead spleen cells (SHS) (IV) and in channel catfish ovary cells (CCO) (III) using undiluted and diluted (10^4) inocula.

Treatment	Virus Code	Day Post-exposure 3	5	7	10	12	15
10^0 CCO	III	5^n -*	2-3+	3-2+	3-2+/++	3-2++/+++	3-2+/++
10^0 SHS	IV	4-1+	4-1+	4-1+	3-2+/++	3-2++	3-2++/+++
10^4 SHS	IV	3-2+	2-3+	3-2+	3-2+	3-2+	3-2+/++
Control		5 -	5 -	5 -	5 -	5 -	5 -

n number of test fish
* see Table 2.3 for lesion grade criteria
number of fish per treatment = 5

Fig. 10.1. Transmission electron photomicrograph (TEM) of 91-97 virus isolate (I) purified after replication in snakehead spleen cells (SHS) (a) and 95-96 virus isolate (III) purified after replication in channel catfish ovary cells (CCO) (b).


a

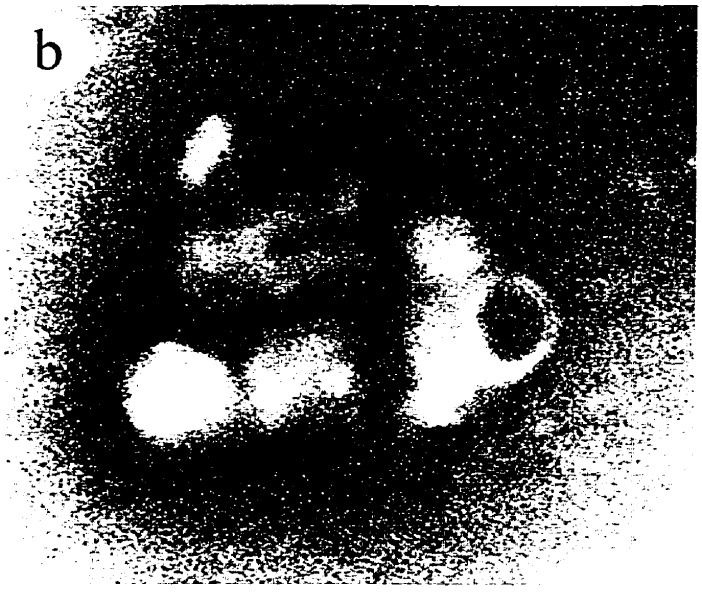

b

Fig. 10.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified virus (silver stain) lanes: virus isolates #91-97 replicated in SHS cells (I), #91-97 replicated in CCO cells (II), #95-96 replicated in CCO cells (III), and low molecular weight protein standards (MM).

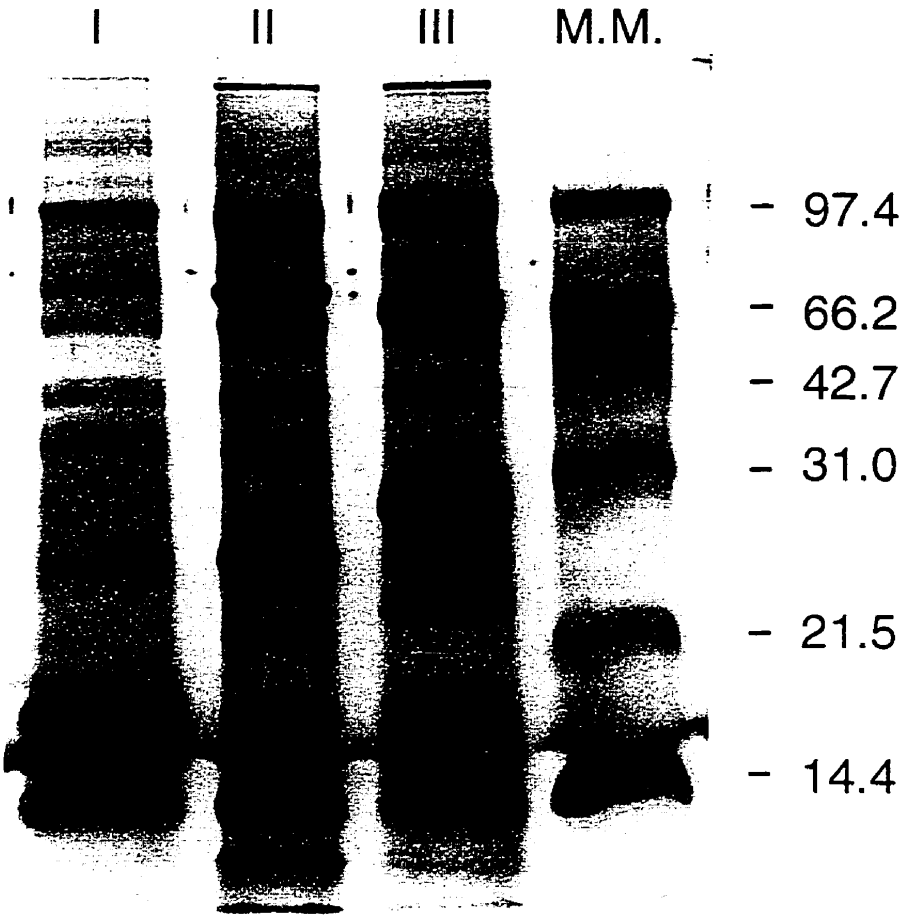
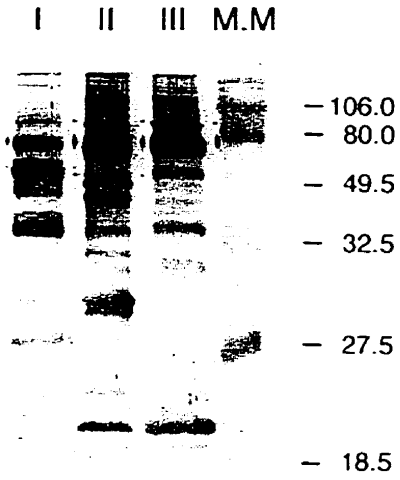
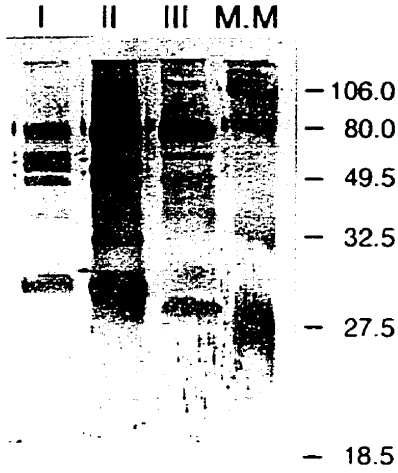

I
II
III
M.M.
- 97.4
- 66.2
- 42.7
- 31.0
- 21.5
- 14.4

Fig. 10.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified virus lanes: #91-97 virus isolate replicated in SHS cells (I), #91-97 replicated in CCO cells (II), #95-96 replicated in CCO cells (III) upon immunoblotting of protein bands after treatment with anti-I serum, anti-II serum, anti-III serum, and low molecular weight protein standards (MM).

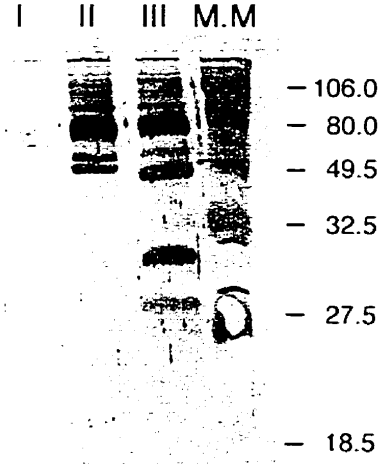
Anti-I serum



Anti-II serum



Anti-III serum



DISCUSSION

The absence of CPE in susceptible cells when viral inocula are undiluted is indicative of the development of defective interfering (DI) viral particles (Huang 1973; Roux *et al.* 1991). These are incomplete virions that compete with non-defective virus for binding sites on the surface of host cells. As a result, DI-infected cells survive. A similar phenomenon has been reported to occur with human and other fish viruses (Nicholson and Dunn 1974; Nicholson and Dexter 1975; Hedrick *et al.* 1978; Macdonald and Yamamoto 1978; Coen and Ramig 1996).

Virus isolate I, developed DI viral particles at undiluted or low dilutions of the virus inocula in SHS and BF2 cells. Lo *et al.* (1990) confirmed that undiluted or low diluted (10^{-1} or 10^{-2}) passages of HB-1, a strain of infectious pancreatic necrosis virus (IPNV), induced autointerference in tilapia cells. These authors attributed this to the presence of DI particles. Other workers also reported that IPNV generated DI viral particles (Hedrick *et al.* 1978; Nicholson and Dexter 1975; Macdonald and Yamamoto 1978).

The development of DI viral particles, however, was not observed when virus I was inoculated into CCO and CFS cells. Clark (1980) reported a similar observation using rabies virus, where the species of the host cell influenced the synthesis of DI viral particles. These observations may, in part, explain why viral isolate I, which was initially isolated in SHS cells, was not pathogenic to naive snakeheads whereas III, isolated and propagated in CCO cells, remained pathogenic (Chapters 7 and 9). This difference in virulence may, at least in part, be attributed to the development of DI viral particles of virus I, when cultured in SHS cells.

White and Fenner (1994) reported that the generation of DI viral particles could result in attenuation of the virus. In fact, DI particles provided protection against influenza virus in 7-week-old Swiss mice and against vesicular stomatitis virus in weanling mice (Jones and Holland 1980). Since the snakehead rhabdovirus I produced DI particles in SHS and BF2 cells, this may be the reason for the nonvirulence of UDRV for experimental snakeheads. This virus was isolated by Frerichs *et al.* (1993) in snakehead cells from EUS fish in Thailand.

Fluctuating incidences of apparent activity of DI viral particles were observed in the present study when viral isolates IV and V were passaged in high multiplicity in SHS cells. This cyclic pattern of DI particle generation was also observed in variable degrees of virulence of the virus *in vivo*. Similarly, Lo *et al.* (1990) showed fluctuation in the production of DI particles of IPNV during 20 serial undiluted passages in tilapia cells. This suggests that DI particle replication was amplified to interfering levels when virus was passaged at high MOI. This cyclic pattern of DI particle production and inhibition of standard virus was reported earlier (Palma and Huang 1974). Huang (1988) suggested further that the low success rate of 30 - 50 % in viral isolations among humans sampled during a full-blown viral epidemic, may be a result of intermittent shedding of DI virions.

Results of the infection experiments in this study showed that continuous undiluted passage of virus isolate V in SHS cells can adversely affect its virulence. In contrast, passage of diluted virus V isolate in the same cells resulted in increased pathogenicity. However, with isolate IV, virulence was not affected by serial undiluted passage in SHS cells. Perhaps the serial passage of the latter virus isolate requires more

continuous passage as undiluted inoculum, beyond the 36th passage in this study. More selective tests need to be conducted to prove this hypothesis.

The SDS-PAGE of the EUS rhabdovirus protein bands clearly shows the similarity of the three test viruses thus providing evidence that these are very closely related, if not the same rhabdovirus. The presence of an additional band in the pathogenic virus (Fig. 11.3, lane III) with an estimated molecular weight of 29 kD may be the virulence-related protein of virus III which was absent in I and II and may be the reason why virus isolate III was pathogenic while II was not.

That virus isolates in this study are closely related are further supported by the results of the Western immunoblotting tests using antisera of the three virus isolates which showed a sharing of antigenic determinants on their structural proteins (Fig. 11.4). It appears that isolates III and I are identical rhabdoviruses. Lilley and Frerichs (1994), likewise, reported the homogeneity and antigenic relatedness among six rhabdoviruses isolated from EUS fish in Thailand, Sri Lanka, Myanmar and Australia.

In SHS cells, the production of DI viral particles was inversely related to temperature, DI activity being detectable after 1, 2, 2 and 5 d of incubation at 30, 25, 20 and 15 °C, respectively. If this occurs, *in vivo*, it at least partially explains why the disease occurs only during the cold season in the Philippines and in other EUS-affected countries and disappears during the warm months of the year, despite the persistence of EUS-associated virus in apparently healthy fish (Chapter 6). The similarity in autointerference observed *in vitro* and *in vivo* with defective interfering IPNV suggested an identical mechanism of DI virus particle production in carrier trout tissues (Hedrick and Fryer 1982).

The implications of DI viral particle generation by the EUS rhabdovirus (ERV) are of utmost relevance to the epizootiological pattern of EUS occurrences. The disease occurs only when rearing water temperatures drop below 25 °C and disappears as soon as water temperatures warm up. However, the virus persists in apparently healthy fish (Chapter 6). Hence, it is hypothesized that during the next annual cold season, DI viral particle generation is inhibited. Instead, complete virus is produced and these induce primary lesions in susceptible fishes culminating another EUS outbreak.

CHAPTER 11

INTERACTION OF THE EUS RHABDOVIRUS (ERV), *AEROMONAS HYDROPHILA* AND *APHANOMYCES* SP. IN THE EXPERIMENTAL INDUCTION OF THE EPIZOOTIC ULCERATIVE SYNDROME (EUS) IN SNAKEHEADS, *OPHICEPHALUS STRIATUS*

INTRODUCTION

In Chapter 9, it was shown that the '95 series viruses induced dermal lesions and mortalities in juvenile snakeheads (*O. striatus*) when injected intramuscularly. Subsequently, it was shown that these viral isolates were similar if not identical to the 91-97 virus isolate, EUS rhabdovirus (ERV) (Chapters 7 and 10). Similarly, the bacterium, *A. hydrophila*, also produces dermo-necrotic lesions after intramuscular injection into catfish (*C. batrachus*) and snakeheads (Chapters 2, 3 and 4).

Among the fungal species of *Aphanomyces*, *Achlya* and *Saprolegnia* that have been isolated from EUS-affected fish, only *Aphanomyces* sp. is able to grow invasively in snakeheads (Callinan *et al.* 1995a; Chinabut *et al.* 1995; Lilley and Roberts 1997; Roberts *et al.* 1993; Willoughby *et al.* 1995). It was reported to cause mycotic granulomatosis in ayu (*Plecoglossus altivelis*) in Japan (Hatai 1994).

Since all of the above types of microbial pathogens have been associated with EUS lesions in fish, it was of interest to observe their combined effect on EUS susceptible fish since it is possible that this disease may be caused by the combined effects of one or more of these pathogens. The interaction of an EUS rhabdovirus and the fungus, *Aphanomyces* has been reported recently (Kanchanakhan 1996; Lilley and Roberts 1997).

To date, experiments involving the three pathogens simultaneously have not been conducted. The objective of this study, therefore, was to experimentally induce lesions in snakeheads (*O. striatus*) using various combinations of these microbes (95-96 virus isolate, *A. hydrophila* and *Aphanomyces* sp.), and to characterize the lesions histologically to determine whether or not they are EUS-like lesions.

MATERIALS AND METHODS

Experimental Fish

Healthy, naive snakeheads, 40 - 80 g wt, were collected from several rivers in Iloilo province and transported to the SEAFDEC, Tigbauan Station. There, the fish were acclimated for at least 1 week in fresh water contained in 1000-liter fiberglass tanks at ambient temperature (25 - 30 °C). The fish were then placed in water of 20 - 22.5 °C for at least 3 d before initiating infection experiments. One fish was placed in each static and aerated 5-liter aquarium containing UV-sterilized freshwater. Except for the bacteria alone treatment, all other treatments were replicated twice using 5 fish per replicate.

Preparation of the Virus Inoculum

The 95-96 virus, isolated from naturally EUS-affected snakeheads, was propagated in CCO cells with MEM-4 without the antibiotic/antimycotic mixture. The supernatant was clarified by low centrifugation and stored at -70 °C until use. Virus titer assays of the viral inocula were determined and estimated to be 1 x 10^7 $TCID_{50}$ per ml according to the method of Rovozzo and Burke (1973) as described in Chapter 7.

Preparation of *Aeromonas hydrophila* Suspension

Pure cultures of *A. hydrophila* isolated from EUS-infected fish from a previous study (Chapter 2) were subcultured on TSA at 25 °C. Overnight cultures of the bacteria were harvested as described in Chapter 4. The cells were resuspended in NSS and diluted to 10^9 cfu per ml. One-tenth ml of this suspension was injected (IM) into each test fish (10^8 cfu per fish). Control fish were injected with NSS only.

Preparation of *Aphanomyces* sp. Inoculum

A pure culture of *Aphanomyces* sp., isolated from EUS-affected fish in Nueva Ecija, Philippines, was obtained from R. Callinan and J. Paclibare (ACIAR Project, Bureau of Fisheries and Aquatic Resources, Quezon City, Philippines). The fungal isolate was subcultured on Czapek Dox medium (Difco) at 25 °C. One-half cm diameter hyphal growth at the edge of a 3-d old culture was cut using a 0.5-cm diameter sterile cork borer and used as inoculum (see below) for the *in vivo* experiment.

Experimental Design of Infection Trials

Challenge treatments consisted of: (1) virus, (2) bacteria, (3) fungus, (4) virus + bacteria, (5) virus + fungus, (6) virus + bacteria + fungus and (7) controls. Naive snakeheads were injected intramuscularly (IM) in the epaxial region of the fish with 0.1 ml of the 95-96 virus suspension ($TCID_{50}$ 10^6 per fish). Five days after viral injection, 0.1 ml of the *A. hydrophila* bacterial suspension was injected IM into the same site as the viral injection for the treatments: virus + bacteria and virus + bacteria + fungus. Fungal inoculation was administered by insertion of approximately 0.5 cm^2 fungal hyphae into the center of the fish lesion or under the scales of unlesioned fish at the site of the virus injection, 5 or 6 d post-viral injection. On the same day, five pieces of the 0.5 cm^2 of fungal hyphae were placed inside a sterile, perforated tissue processing cassette and placed into the rearing waters of the treatments: fungus, virus + fungus, and virus + bacteria + fungus. All experiments were conducted in water at 20 - 22.5 °C .

Monitoring of Experimental Fish

All fish were examined daily for mortality and development of EUS-like lesions. Lesions were characterized according to the criteria in Table 2.3.

Histopathology

Tissue samples of the skin/muscle lesions were collected from the experimental snakeheads at d 6 - 15 post-viral injection, at d 3 - 4 post-bacterial injection, and at d 5 - 10 post-fungal inoculation. They were fixed in Bouin's solution (Humason 1979) for 24 h

then changed to 70 % ethanol until processing. Tissue samples were decalcified for a week before being subjected to the standard histological processing procedure. De-waxed histological sections of 5 um thickness were then stained with hematoxylin-eosin (H & E) following the method of Humason (1979). Other sections were also stained with Gomori methenamine silver (GMS), periodic acid-Schiff, Giemsa, and Brown and Brenn Gram's stain (BBG) using the methods described by Luna (1968). Stained sections were evaluated by light microscopy.

RESULTS

Intramuscular injection with the virus alone resulted in early dermal lesion development (+) by d 3 at the site of injection (Table 11.1). The induced lesion gradually intensified to the +++ grade by d 8. When *A. hydrophila*, was also injected (IM) at the same site 5 d after the virus injection, dermal lesion development was more rapid, yielding a most severe dermomuscular lesion (++++) in 72 h. When injected alone, *A. hydrophila*, induced a + lesion 24 h after injection, a +++ lesion in 48 h and a ++++ lesion 72 h post-injection. Fungal inoculation with *Aphanomyces* sp. alone, induced only + to ++ dermal lesions. It was noted that in the virus only, bacteria only and virus+bacteria treatments, all test fish developed dermonecrotic lesions while in the fungal experiments, two of five test fish in both replicates did not exhibit any dermal pathology.

Among experimental fish with multiple inocula consisting of the 95-96 virus and *A. hydrophila*, dermal lesions started to appear at d 3 post-viral injection. These

Table 11.1. Interaction at 21 - 24 °C of the 95-96 virus isolate (10^6 $TCID_{50}$ per fish) injected intramuscularly (IM), *Aeromonas hydrophila* (10^9 cfu per fish) injected IM, and *Aphanomyces* sp. by subdermal insertion (0.5 cm^2 diameter mycelia per fish plus 4 x 0.5 cm^2 diameter mycelia per aquarium) in causing lesion development in naive snakeheads (*Ophicephalus striatus*).

Treatment	No. of fish	Trial no.	Days Post-exposure 3	4	5	6	7	8	9	10	11	12
Virus	5	1	1[n]-*4+/++	1+4+/++	1+4+/++	1+4+/++	1+4+/++	1-4+/+++	1-4+/+++	3+/++	3+/+++	3+/+++
	5	2	5 +	5+	5+/++	4+/++1d	4+/++	4+/++	3+/++1d	2-1+++		
Virus + bacteria	5	1	5 +	5 +	1-4+/++	1-3+/++1d	4+/+++	4++/+++	4++/++++	2++/++++2d	2++/++++	2++/++++
	5	2	5 +	5+	5+/++	3+/++2d	2+/+++1d	2++/++++	2++/++++	1++1d		
Virus + bacteria + fungus	5	1	5 +	5 +	4+/++1d	4+/++	4++/+++	4+++	2+++2d	2+++	2+++	2+++
	5	2	5 -	5+	5+/++	4+/+++1d	4++/+++	3+/+++1d	3+/+++	3+/+++		
Virus + fungus	5	1	1-4+	3+2d	3+/++	3+/++	3+/+++	3+/+++	3+/+++	3+/+++	1++2d	1++
	5	2	5 +	5+	5+/++	5+/++	5+/+++	5+/+++	5+/+++	1-3+/++1d		
Fungus	5	1	ns	ns	ns	ns	4-1+	2-3+	2-3+	2-3+	2-3+	2-1+
	5	2	ns	ns	ns	3-2+	2-3+	2-3+	2-3+/++	2-3+/++		
Bacteria**	5	1	ns	ns	ns	ns	5 +	5++/+++	5++/++++	3+++/++++	3++++	1++++
Control	5	2	5 -	5 -	5 -	5 -	5 -	5 -	5 -	5 -	5 -	5 -
	5	2	5 -	5 -	5 -	5 -	5 -	5 -	5 -	5 -	5 -	5 -

n number of fish

* see Table 2.3 for lesion grade criteria

ns fish stocked but treatment not started

** see Table 2.8 for related experimental data

developed to ++++ lesions by d 8 post-viral injection or d 2 post-bacterial injection. In the virus plus fungus treatment, initial lesions (+) were observed at d 3 and these developed gradually to +++ lesions by d 7. In the virus plus bacteria plus fungus treatment, lesion development followed a similar pattern to that of the virus plus fungus treatment. Mortalities, however, were observed only in treatments in which the 95-96 virus was injected. None of the control fish developed any lesions or mortality.

Histopathological examination of experimental fish showed that those injected (IM) with the 95-96 virus alone consistently manifested slight or moderate to severe cellular, inflammatory infiltration of the muscle fibers (Fig. 11.1 b, c). Slight inflammation was observed among fish samples with + lesions while moderate to severe inflammation was observed among fish sampled at d 8 to 15. It is interesting to note that the inflammatory response was observed also among samples taken from the periphery of the dermal lesion. Some fish samples also showed slight, moderate and severe dermal hyperplasia as well as slight to moderate muscular necrosis.

Fish injected with *A. hydrophila* that developed +++ to ++++ lesions consistently exhibited moderate dermal hyperplasia and moderate to severe degenerative necrosis of the hypodermis and muscle fibers plus moderate edema (Fig. 11.1 d, e). In a few samples, liquefactive necrosis and moderate inflammation were also observed.

Fish treated with *Aphanomyces* sp. developed focal granulomas containing the fungal hyphae with or without necrosis (Fig. 11.1e). Otherwise, the overall histological profile appeared normal.

The virus plus bacteria treatment consistently showed moderate dermal

Fig. 11.1. (a) Sham-injected juvenile snakeheads (*Ophicephalus striatus*) showing normal epidermis (E), dermis (D), hypodermis, and muscle fibers (M). H&E, x100

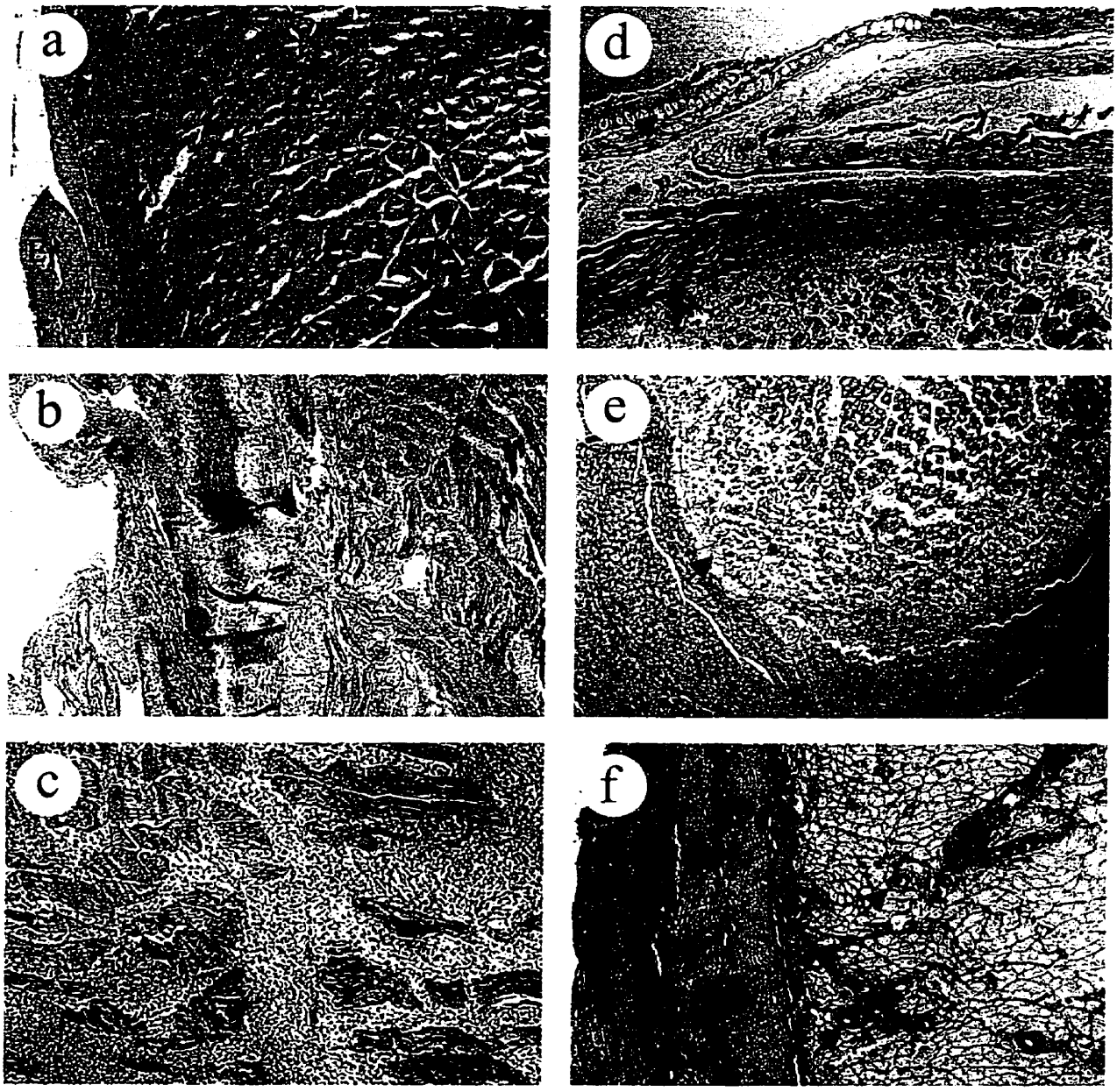
(b) Skin/muscle lesions (+++) in juvenile snakehead, 9 d after IM injection with 95-96 virus isolate resulting in hyperplastic epidermis, degenerated hypodermis and severe inflammatory infiltration of the myofibrils. H&E, x200

(c) High magnification of Fig. 11.1(b) showing severe myositis. H&E, x400

(d) Juvenile snakehead 4 d after IM injection with *Aeromonas hydrophila* causing hyperplastic and spongiotic epidermis and necrosis of the hypodermis and muscle fibers. H&E, x100

(e) High magnification of muscular tissue in Fig. 11.1 (d) exhibiting liquefactive necrosis. H&E, x400

(f) Multifocal granuloma in the epidermis, dermis and myofibrils induced in juvenile snakehead 10 d post-exposure to *Aphanomyces* sp. GMS, x100


a
d
b
e
c
f

hyperplasia, moderate to severe myonecrosis and slight or moderate to severe myositis (Fig. 11.2a). Some fish samples exhibited moderate edema in the muscular fibers and necrosis of the hypodermis. In the virus plus fungus treatment, consistent signs were slight epidermal hyperplasia, slight to severe dermal hyperplasia, myonecrosis and myositis as well as hypodermal necrosis (Fig. 11.2b). Among fish given the three microbial inocula, moderate to severe muscular necrosis and inflammation was most consistent (Fig. 11.2c). None of the control fish showed any histological signs of pathology.

DISCUSSION

The results of this study indicate that the 95-96 virus, *A. hydrophila* and *Aphanomyces* sp. can separately induce dermal lesions in snakeheads at the site of inoculation. Severity of the lesion development, however, varied with the pathogen, time post-inoculation and with whether more than one infectious organism was administered. *Aeromonas hydrophila* consistently produced the most rapid onset of lesions (+) 24 h post-injection and the most severe dermonecrotic muscular lesions (++++) 72 h after treatment. Similar results were obtained in earlier studies (Chapters 2, 3 and 4).

The histological profile of fish with +++ and ++++ lesions consistently showed necrosis of the musculature and hypodermis, dermal hyperplasia and edema. The presence of bacteria was not evident either at the lesion center or at the lesion periphery.

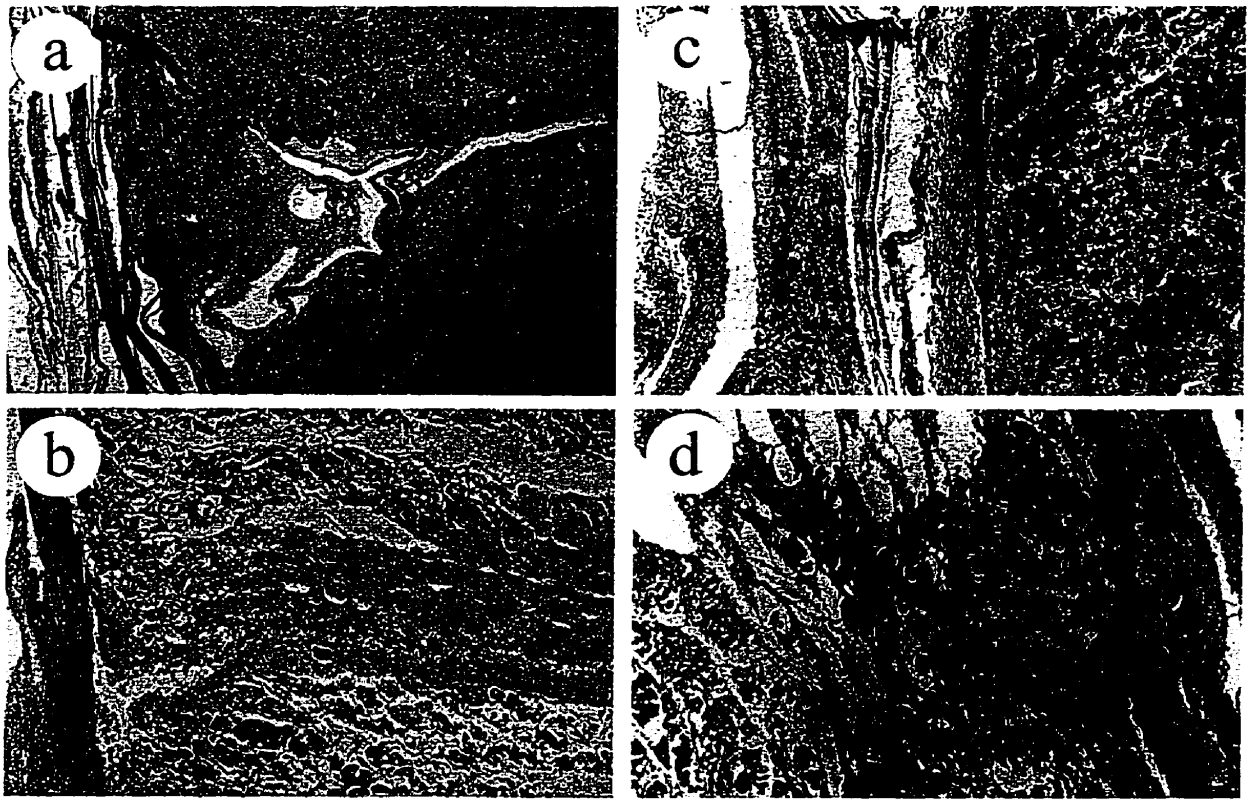
Brenden and Huizinga (1986) reported similar histopathological findings among goldfish (*Carassius auratus*) intramuscularly injected with *A. hydrophila* and by

Fig. 11.2. (a) Site of injection in the skin/muscles of juvenile snakehead (*Ophicephalus striatus*) showing slight spongiosis of the epidermis, hyperplastic dermis and liquefactive necrosis of the muscle fibers following exposure to the 95-96 virus *and A. hydrophila* for 9 and 5 d, respectively. BBG, x 40

(b) Hyperplastic and degenerative changes in the epidermis and musculature with concomitant edema in juvenile snakehead after multiple inoculation with the 95-96 virus and *A. hydrophila*. H&E, x100

(c) Epidermal dermatitis with inflammatory infiltration of the hypodermis and myofibrils following exposure of the juvenile snakehead to the 95-96 virus and *Aphanomyces* sp. H&E, x100

(d) Epidermal dermatitis, hyperplastic dermis and severe inflammation of the hypodermis and musculature with severe granulomatous necrosis of the myofibrils in juvenile snakehead after multiple inoculation with the 95-96 virus, *A. hydrophila* and *Aphanomyces* sp. H&E, x100


a
c
b
d

Huizinga *et al.* (1979) among largemouth bass (*Micropterus salmoides*) exposed to water-borne *A. hydrophila*. The former workers also reported the absence of bacteria in Gram stained tissues more than 1 h after treatment. The histopathological findings may be attributed to the production of extracellular proteins or cytotoxins by *A. hydrophila*, which may account for the progressive skeletal muscle necrosis at the site of inoculation (Yadav *et al.* 1992; Leaño *et al.* 1996).

The 95-96 virus induced slight (+) lesions by d 3 which gradually developed to +++ lesions by d 8 following IM injection. As in previous studies (Chapter 9), no severe lesions (++++) were observed.

Deaths were observed only among treatments containing the 95-96 virus isolate as inoculum. Microscopic analyses of the tissues surrounding the site of viral injection showed the consistent appearance of slight to severe inflammatory reaction.

Inflammatory response was reported among wild largemouth bass artificially infected with an iridovirus (Plumb *et al.* 1996). Mortalities were observed only in this treatment although previous studies have shown that bacterial infection with *A. hydrophila* also can cause mortalities (Chapter 2). A viremia is most probable after viral entry into the fish and earlier studies showed the presence of the virus in visceral organs (Chapter 6 and 8).

Fungal treatment showed the invasive tendency of the *Aphanomyces* sp. hyphae. These penetrated the muscle fibers and stimulated granuloma formation about the invading microbe. Similar results were reported previously in infection experiments with snakeheads (Roberts *et al.* 1993; Chinabut *et al.* 1995). However, only 3 of 5 fish inoculated with the fungus alone in this study, exhibited + / ++ lesions. In addition, Lilley

and Roberts (1997) reported that in all cases of experimentally infected *Aphanomyces* sp., mild traumatic damage such as sarcoplasmic degeneration, hemorrhage and cellular inflammatory infiltration were observed. Hence, fungal pathogens are most likely opportunistic, secondary invaders in the pathogenesis of EUS.

In artificial multiple infections with both the 95-96 virus and *A. hydrophila* as inocula, the external lesion development and histopathological observations paralleled those treated with either virus or bacteria. Epidermal hyperplasia was observed in addition to dermal hyperplasia, hypodermal necrosis, as well as myonecrosis and myositis. In bacterial diseases such as vibriosis, liquefactive necrosis of the muscle can be pronounced (Ferguson 1989). However, in the virus plus fungus treatment, the lesion induced initially by the viral injection did not intensify with additional inoculation of *Aphanomyces* sp. Histologically, no fungal hyphae were detected in the experimental fish tissues. Hence, the dermal pathology observed can largely be attributed to the effect of 95-96 virus alone. In contrast, Kanchanakhan (1996) reported that the combination of the rhabdovirus and the fungus, *Aphanomyces*, induced more severe EUS-like lesions in snakeheads than the fungus alone.

Among fish treated with a combination of the three pathogens, muscular necrosis, inflammation and hypodermal necrosis were most commonly observed. No fungal granulomatous reaction was detected.

This is the first report showing the interaction of the three microbial pathogens: ERV, *A. hydrophila* and *Aphanomyces* sp. Histopathological findings in artificially infected snakeheads were largely as observed in the natural EUS-affected fish but less severe. Among natural EUS-affected fish, necrotizing and mycotic granulomatous

inflammation were reported as the typical histological profile (Cruz-Lacierda 1995; Mohan and Shankar 1995). Among the three test pathogens in this study, however, only *A. hydrophila* caused ulcers in artificially infected fish (Chapter 2). Since ulcerated lesions are common features of moderate and severe cases of EUS, the ability of *A. hydrophila* to induce ulcers strongly implicates the involvement of this bacterium in the pathogenesis of EUS. Moreover, Roberts *et al.* (1994) reported that death among EUS fish was associated with severe generalized edema and secondary bacterial septicemia.

A. hydrophila and *Aphanomyces* sp. have been consistently associated with naturally-affected EUS fish, and are also found in rearing waters and sediments (Chapter 2; Llobrera and Gacutan 1987; Roberts *et al.* 1993; Callinan *et al.* 1995b). They are likely to act as secondary invaders of the primary lesion induced by the ERV. Fish epizootics involving both bacterial (*Vibrio*) and viral (reovirus) etiologies have also been reported in cultured turbot (*Scophthalmus maximus*) in Spain (Lupiani *et al.* 1989). Likewise, the involvement of fungal pathogen/s among naturally affected EUS snakeheads has been reported extensively (Frerichs *et al.* 1989b; Callinan *et al.* 1995a).

However, it must be borne in mind that ERV can only cause dermal lesions and mortalities at low temperatures (19 - 25 °C) whereas *A. hydrophila* and *Aphanomyces* sp. are pathogenic at both low and high (31 °C) water temperatures (Chapter 2 and 9; Chinabut *et al.* 1995). In addition, in a temperature-growth relationship study on eight isolates of *Aphanomyces* spp. from EUS fish in Thailand, the colony radius of the fungi were consistently 1.3 – 8 mm larger when incubated at 31 °C than those incubated at 18 and 21 °C. Thus, if either *A. hydrophila* or *Aphanomyces* spp. is the primary pathogen,

EUS outbreaks should not only occur during the cold months of the year in the Philippines and other affected countries.

On the basis of the present results, it appears that ERV is the primary agent of EUS, and it induces the initial dermomuscular lesion. Thereafter, this primary infection is complicated by secondary infection with either the bacterium, *A. hydrophila* and/or the fungus, *Aphanomyces* sp. EUS fish that develop the primary lesion during an epizootic either die or survive and manifest the superimposed secondary pathology caused by bacterial and/or fungal infection. The virus may predispose its host to secondary bacterial infection by immunosuppression. Since bacterial infection by *A. hydrophila* causes the development of sloughed or ulcerated musculature, this dual cause is the most probable scenario during the annual outbreak of the EUS.

CHAPTER 12

GENERAL DISCUSSION

Current information about the epizootiology of EUS must be considered in the overall analyses of the disease. Firstly, metazoan and protozoan parasites associated with EUS fish are unlikely pathogens of the disease (Lilley *et al.* 1992; Roberts *et al.* 1994). Secondly, pesticides and non-biological factors have been shown not to cause the disease (Roberts *et al.* 1994; Phillips 1994). Thirdly, there is a consistent correlation of the appearance of EUS with the occurrence of relatively cooler water temperatures which prevail during December to February in the Philippines each year (Palisoc 1990; Phillips 1994). Lastly, the expanding geographical pattern of the disease in each affected country strongly suggests a microbial etiology for EUS. Thus, there have been several investigations of the pathogens that are associated with EUS.

My results indicate that ERV is the primary etiologic agent of EUS for the following reasons:

(1) It was the only virus isolated from EUS-affected fishes and it was frequently detectable in such fish (Chapter 6 and 8).

(2) EUS could be transmitted to healthy naive catfish and snakeheads from EUS-affected or apparently healthy but virus-positive fish. It was also transmitted from virus-contaminated waters to naive fish under natural simulated conditions during the EUS enzootic months. ERV was subsequently isolated from these experimentally infected naive fish, but not from unexposed naive fish (Chapter 8).

(3) The replication temperature requirements of ERV corresponded with the seasonal water temperature ranges occurring during natural EUS outbreaks.

(4) ERV induced moderate dermomuscular lesions in juvenile snakeheads by IM injection at 20 - 22 °C but not at 28 - 32 °C. It also caused mass mortalities of snakehead fry and fingerlings following bath exposure to the virus.

(5) Among the three important pathogens (ERV, *A. hydrophila* and *Aphanomyces* sp.) consistently associated with EUS-affected fish, only ERV has shown temperature sensitivity in terms of replication and pathogenicity consistent with natural EUS outbreaks.

(6) ERV also demonstrated DI particle generation. Its occurrence is inversely related to temperature and correlated with the outbreak pattern of natural EUS epizootics.

ERV was frequently isolated from EUS-affected fishes in the Philippines. The success of viral isolation improved to a 35 % recovery rate in this study (Chapter 6), following the use of 1:10 dilution of tissue filtrates in place of 1:50 – 1:100 dilutions. Virus isolations could be made either from muscle lesions or viscera of moderately lesioned fish. Apparently healthy fish even harbored ERV, which persisted in enzootic areas during non-epizootic periods (Chapter 6). Virus was isolated from several species of EUS-affected fishes, confirming the wide host range of ERV.

Hedrick *et al.* (1986) reported the isolation of a birnavirus, Frerichs *et al.* (1989a), Kasornchandra *et al.* (1992), and Lilley and Frerichs (1994) several rhabdoviruses, and Frerichs *et al.* (1989b) a reovirus from EUS-lesioned fish. The isolations of the birnavirus and the reovirus were reported only once while the rhabdovirus has been very frequently reported from EUS-affected fishes.

The importance of the rhabdoviruses associated with EUS as the most probable viral pathogen is emphasized by the related studies conducted. Lilley and Frerichs (1994) reported seven rhabdovirus isolates (BPV, 02, 19, 20E, A4, SL11 and T9204) which were classified as a structurally homogenous and antigenically related group known as UDRV. Isolate T9204 was morphologically and an antigenically different and perhaps similar to the SHRV described by Kasornchandra *et al.* (1991). Morphological, cytopathological and serological studies, characterized the genus of SHRV as lyssaviruses and the UDRV as vesiculoviruses (Kasornchandra *et al.* 1992). Moreover, in the 1993-94 EUS outbreaks in Thailand, nine rhabdovirus strains were isolated, and proposed as serotypes Sh and Ud (Kanchannakhan 1996).

ERV replicated to titers of 10^5 to 10^7 $TCID_{50}$ per ml in cell culture at 15 to 30 °C with the highest titer attained at 15 °C. The lower replication temperature corresponds with the water temperature ranges prevalent during the EUS outbreaks. Also, based on laboratory results, the virus is more likely to be quickly inactivated in the aquatic environment when exposed to temperatures above 30 °C. Such temperatures occur during the non-EUS enzootic months.

Transmission electron microscopy of the purified I and III EUS viral isolates showed that both had the bullet morphology typical of the rhabdoviruses. SDS-Page

protein bands of the purified I, II and III EUS viruses revealed similarity in the protein band profile except for the III isolate which exhibited an additional band with an approximate molecular weight = 29 kD. This additional protein is perhaps a virulence related protein band. Immunoblotting of these protein bands revealed that the virus isolates I, II and III shared antigenic determinants on their structural proteins, and hence, are likely identical (or very closely related) rhabdoviruses.

The SDS-Page of the protein bands of the EUS rhabdovirus clearly shows the similarity of the three test viruses, thus providing evidence that the three rhabdoviruses are identical or essentially identical. The additional band in the pathogenic virus (lane III) with an estimated molecular weight of 29 kD is hypothesized to be the virulence protein of the #95-96 EUS virus. This may be why the #95-96 isolate is pathogenic whereas the #91-97 isolate is not. These findings are further supported by the results of the Western immunoblotting tests done using the specific antiserum of the three virus isolates showing a sharing of antigenic determinants on their structural proteins. Lilley and Frerichs (1994), likewise, reported the homogeneity and antigenic relatedness of six EUS rhabdovirus isolates from Thailand, Sri Lanka, Myanmar and Australia. Hence, the name EUS rhabdovirus (ERV) is proposed.

The horizontal transmission experiments of EUS under simulated natural conditions revealed that ERV can be transmitted to naive snakeheads by cohabitation with EUS individuals or apparently healthy (but virus-positive) snakeheads in lakewater or aquifer water; or in contaminated waters without fish; and to catfish by cohabitation with EUS-affected snakeheads in lakewater with 100 % morbidity rates. This was shown by the onset of EUS lesions among naive fish within 6 d of exposure and by the recovery

of virus from the exposed naive fish but not from the unexposed ones. The differences in time of onset of lesion development was directly related to the viral inoculum size, thus naive fish exposed to EUS or apparently healthy fish exhibited a shorter incubation period prior to lesion development compared with those fish exposed to contaminated lakewater alone. Virus titer in the lakewater was probably lower than that in virus-positive "source" fish, and therefore, the challenged fish had exposure to less virus, thus requiring a longer incubation period of 10 more days to manifest lesion development. Hence, the incubation period of EUS may range from 6 to 14 d depending upon the source of the virus. The pathogenesis of the disease, however, follows a similar path: severe lesion development occurred at 5 d after onset of initial lesions. Deaths occurred 5 d after severe EUS signs were detected with more than 50 % mortalities occurring 8 d after onset.

Natural transmission of EUS appeared to be associated with low water temperatures, which prevail during the EUS season. That viral infectivity of ERV persisted longer at 20 °C than at 30 °C in a cell-free environment (Chapter 7), explains in part why EUS outbreaks predominantly occur during the cold season in the tropics. Since both male and female fish, and even gravid females, were positive for virus, and taking into consideration the isolation of virus from the ovaries of EUS-affected fish, suggests that vertical transmission of the ERV may also occur.

Infection experiments using pure cultures of the 95-96 isolate corroborated the more natural transmission experiments and resulted in dermal lesion development (graded + to +++) 3 to 10 d following IM injection of naive juvenile snakeheads reared at 20 - 22.5 °C. As expected, no lesion developed at 28 - 32 °C. Moreover, the injected virus was consistently reisolated from the 20 - 22.5 °C but not from the higher temperature treated

fish. The lesions progressed to moderate lesions by d 10 - 12 but not to the deep ulcers seen in snakeheads with natural EUS. The virus caused lesion development in the majority of IM-injected fish and required a dose of at least 10^3 $TCID_{50}$ per fish. Moreover, the onset of mild lesions (+) and progression of lesion development to +++ lesions among artificially-infected fish in 5 and 10 to 12 d, respectively, (Chapter 9) were comparable to the relative incubation period observed among naive snakeheads exposed to EUS fish under naturally-simulated conditions. Naive snakeheads naturally exposed to EUS virus via diseased or apparently healthy fish carriers developed lesions in 6 and 9 to 11 d, respectively (Chapter 8).

Virus virulence was not correlated with the organ source of the virus or with the passage frequency of the virus in cell cultures. Among snakehead juveniles, only the IM and SD injected test fish developed lesions while virus given orally by intubation or by immersion exposure did not induce lesions or mortalities. Among snakehead fry and fingerlings, however, immersion exposure to the virus caused heavy mortalities. IP injections of the virus did not induce lesions in the test fish but caused mortalities among the majority (75 %) of virus-injected fish. IP injections must have provided the virus with direct access to the visceral organs of the injected fish and caused fatal damage to the fish. Cruz-Lacierda (1995) reported histopathologies in different visceral organs of snakeheads exposed to EUS-affected fish. Similarly, findings included in Chapter 8 indicate the wide range of visceral organs in which ERV appeared to invade.

Viral virulence was also related to fish size as naive snakehead fry and fingerlings exposed to the ERV by bath manifested significant mortalities ($p<0.01$) by d 4 and 5 with

no apparent lesions. Hence, ERV could have devastating effects in hatcheries when temperatures are optimum for viral replication.

Generally, the incubation periods and speed of lesion advancement among naturally exposed and artificially infected fish were similar. Mean mortalities were significantly higher in the IM virus-injected fish (72 %) compared to the mean mortalities of non-infected fish (33 %). Fishers and fishfarmers in the Philippines (pers. comm.) observed that dead EUS-lesioned fishes were often seen floating on the surface of lake or pond waters during the EUS season.

The generation of DI particles by ERV is a critical factor in its virulence. DI occurrence in cell cultures was inversely related to incubation temperature and viral dilution. Also, DI particle production showed a cyclic pattern. Hence, when viral suspensions rich in DI particles were injected intramuscularly (IM) into naive snakeheads, no dermal lesions were induced while the serially diluted inoculum or virus propagated in CCO cells (which produce only intact virus) produced dermal lesions. The virus was recovered from the viscera of test fish for at least 12 d following exposure, which indicates that the virus remained viable in that inoculated fish.

The generation of DI viral particles and environmental temperature may be triggering factors in the attenuation and outbreak of EUS, respectively. DI viral particles developed at undiluted or at lower dilutions of the virus inocula in SHS or BF2 cells resulting in autointerference. In SHS cells, the replication of DI viral particles was inversely related to temperature since they were detectable after 1, 2, 2 and 5 d of incubation at 30, 25, 20 and 15 °C, respectively, using undiluted viral inocula. Thus, during the EUS epizootic when water temperature range from 18 - 25 °C, the replication

of complete viruses is enhanced, resulting in increased viral population capable of triggering dermal/muscle lesions.

The development of DI virai particles was not observed when the EUS virus was inoculated into CCO and CFS cells. Hence, the 95-96 virus isolate, which was isolated and primarily propagated in CCO cells, was pathogenic to naive snakeheads while the 91-97 virus isolate which was isolated and primarily propagated in SHS cells was not pathogenic to naive snakeheads. This difference in virulence may be attributed to the development of DI viral particles in the course of propagating the 91-97 virus in SHS cells. White and Fenner (1994) reported that the generation of DI viral particles could result in attenuation of the virus. Corollary to this development, are observations by fish farmers that the catfish catch has dropped considerably compared with that of snakeheads (pers. comm.). The fact that catfish cells do not cause DI particle generation may help to explain these observations.

Histopathological examination of the virus-injected tissues of experimental snakeheads showed predominantly severe cellular inflammatory infiltration of the myofibrils about the dermal lesion. Fish injected with *A. hydrophila* consistently exhibited moderate dermal hyperplasia, moderate to severe degenerative necrosis of the hypodermis and muscle fibers, and moderate edema. *Aphanomyces* sp.-treated fish developed focal mycotic granulomas with or without inflammation or necrosis.

The virus plus bacteria treatment resulted in exacerbation of the viral lesion to the +++ to ++++ stage. Histologically, moderate dermal hyperplasia, moderate to severe myonecrosis and slight to severe myositis were consistently observed. In general, the

histopathology observed in experimentally infected snakeheads was similar to the histopathological profile of naturally affected snakeheads but at a lesser severity.

The highest success rate of positive virus isolations among EUS-affected fishes with grade II lesions further sustains the hypothesis that intensification of lesion development, such as that observed in Grades III and IV EUS lesioned fish, is probably a result of a superimposed secondary bacterial infection due to *A. hydrophila* (Chapters 2, 3 and 4) or due to fungal infection with *Aphanomyces* sp. (Roberts *et al.* 1993).

Dermal lesions that developed from ERV injection, did not intensify when followed by additional inoculation with *Aphanomyces* sp. Histologically, no fungal hyphae were detected in the experimental fish. Hence, the dermal pathology observed was largely attributed to the effect of ERV alone. In addition, only 3 of 5 fish inoculated with the fungus alone exhibited lesions, and the lesions were relatively mild (+ to ++). Among fish treated with a combination of the three pathogens, muscular necrosis, inflammation and hypodermal necrosis were most commonly observed, a reaction attributable to the viral and bacterial portion of the inoculum.

It is clear that *A. hydrophila* exacerbated a pre-existing dermomuscular lesion induced by ERV (Chapter 11). Previous studies reported that total bacterial counts of skin and muscle/dermal lesions were 1.22×10^3, 1.40×10^5, 5.31×10^5 and 1.14×10^7 cfu per g of tissue in apparently healthy, slightly lesioned, moderately lesioned and severely EUS-lesioned snakehead samples, respectively (Chapter 2). *A. hydrophila* is the bacterium most consistently associated with EUS-affected fishes (Chapter 2; Boonyaratpalin 1989; Karunasagar et. al. 1994; Llobrera and Gacutan 1987; Pal and Pradhan 1990; Torres *et al.* 1990). It was shown also that *A. hydrophila* induced the most

rapid onset of lesions (+) (as early as 24 h post-injection) and the most severe dermonecrotic muscular lesions (++++) (by 72 h after treatment) in catfish and snakeheads. The dermomuscular necrotic lesions resembled the EUS Grade IV lesions (Chapter 2, 3 and 4; Karunasagar *et al.* 1994; Pal and Pradhan 1990). The rapid onset and development of acute dermomuscular necrotic lesions within 24 h of inoculation of the pure culture of *A. hydrophila* isolate was dose related (Lio-Po *et al.* 1996). Subsequently, deep ulcers similar to EUS lesions observed in natural outbreaks appear (as early as 72 h post-injection). Leaño *et al.* (1996) recently showed that extracellular proteins (ECP) secreted by *A. hydrophila* cause this effect. The histological profile of experimentally infected fish with +++ and ++++ lesions was characterized by necrosis of the musculature and hypodermis, dermal hyperplasia and edema. The experiments also showed that *A. hydrophila* could cause severe necrotic lesions at both low (22 - 25 °C) as well as high temperatures (30.5 - 31 °C). These findings are significant since EUS outbreaks in the Philippines occur annually only when water temperatures fall below 25 °C (during December to February).

Fungal hyphae have been consistently observed in tissue sections of EUS-affected fishes showing severe necrosis of the epidermis, dermis and skeletal muscles and chronic granulomas enveloping degenerating fungal hyphae (Chinabut *et al.* 1995; Cruz-Lacierda 1995; Mohan and Shankar 1995; Roberts *et al.* 1994). In my experiments, *Aphanomyces* sp. when inoculated alone, induced only mild lesions and histopathology in 3 of 5 snakeheads in two trials, at 20 - 22.5 °C. Fungal challenge showed the invasive ability of the test *Aphanomyces* sp. hyphae, which penetrated the muscle fibers and stimulated granuloma formation about the invading microbe. Similar results were reported

previously in infection experiments with snakeheads (Roberts *et al.* 1993; Chinabut *et al.* 1995; Lilley and Roberts 1997). Also, Chinabut *et al.* (1995) reported infection experiments successfully inducing lesions to snakeheads after injecting *Aphanomyces* sp. at temperatures of 19, 26 and 31 °C. Lilley and Roberts (1997), however, concluded that *Aphanomyces* sp. induced only mild histopathology in artificially infected snakehead fingerlings and did not consider this as the primary cause of EUS.

Achlya sp. and *Aphanomyces* sp. were isolated from EUS-affected fish (Lilley and Roberts 1997; Paclibare *et al.* 1994). Paclibare *et al.* (1994) cultured *Aphanomyces* sp. at 28 - 30 °C implying that the fungus can thrive in natural conditions other than the epizootic season for EUS. Despite this, the disease occurs only during the cold months of the year when the temperature drops below 25 °C. It is also interesting to note that Willoughby (1995) reported that *Aphanomyces* sp. found in live, EUS-affected fish were not viable and that the fungus thrived only in muscle tissues of dead fish. These findings strongly indicate that this fungus is usually only a secondary pathogen in fish (Roberts 1989; Post 1987).

Aphanomyces sp. may be pathogenic to snakeheads but its virulence is not temperature-dependent (Roberts *et al.* 1993). Since *in vitro* growth of 41 isolates of *Aphanomyces* spp., 5 isolates of *Achlya* spp. and 12 isolates of *Saprolegnia* spp. from EUS, RSD and other fungal-infected fish worldwide, showed optimal colonial growth at 26 and 30 °C than those incubated at 6,10, 14, 18, and 22 °C (Lilley and Roberts 1997), it is not likely that any of these fungi serve as primary pathogen of EUS. ERV, on the other hand, is only pathogenic at low temperatures (20 - 22.5 °C) (and not at 28 - 32 °C). *A. hydrophila*, is another opportunistic pathogen that has been shown to induce tissue

necrosis in snakeheads at both low (22 – 25 °C) and ambient temperatures (30.5 – 31 °C) . Thus, if either *A. hydrophila* or *Aphanomyces* spp. were the primary pathogen of EUS, outbreaks should not only occur during the cold months of the year in the Philippines and other affected countries.

On the basis of the present results, it is concluded that the EUS virus is the primary agent of EUS inducing initial lesion. Thereafter, this primary infection is abetted/worsened by secondary infection with the bacterium, *A. hydrophila,* and/or the fungus, *Aphanomyces* sp. Since bacterial infection by *A. hydrophila* causes sloughing of the ulcerated musculature, this results in the deep ulcers seen in EUS. It is most likely that the fungal infection sets in after the necrotic lesions have developed as a result of secondary infection with *A. hydrophila*. This is the most probable scenario during the annual outbreak of the EUS.

The present experiments have shown that temperature plays an essential role in the pathogenesis of the disease. ERV can only cause dermal lesions at 20 - 22.5°C and not among those fish reared at 28 - 32 °C, whereas *A. hydrophila* and *Aphanomyces* sp. are pathogenic at both low and high (31 °C) water rearing temperatures (Chapter 2 and 9; Chinabut *et al.* 1995). This corresponds with natural fresh water temperature in various aquatic systems during EUS outbreaks in the Philippines and in other EUS-affected countries in SouthEast and East Asia. The higher temperature range during months when EUS do not occur is not optimum for propagation of ERV (Chapter 7) and promotes DI particle generation. Hence, the susceptible fishes do not express the disease. Thus, this study strongly suggests that the rhabdovirus is the primary causative agent of EUS with *A. hydrophila* and *Aphanomyces* sp. as secondary and tertiary pathogens, respectively.

Overall, this study covered first reports on:

(1) *Aquaspirillum* sp. associated with diseased fish.

(2) Establishment of cell cultures from catfish heart (CFH) P-51, catfish liver (CFL) P-8, catfish spleen (CFS) P-54, snakehead gonad (SHG) P-26, snakehead heart (SHH) P-22, snakehead kidney (SHK) P-19, snakehead liver (SHL) P-49 and snakehead spleen (SHS) P-74.

(3) Detection of ERV in apparently healthy snakeheads during the EUS epizootic.

(4) Detection of viral neutralizing antibodies among apparently healthy and EUS-affected snakeheads from EUS enzootic areas.

(5) Data on daily prevalence values of EUS among snakeheads over a 1 month epizootic period.

(6) Successful horizontal transmission of EUS and ERV from EUS-affected snakeheads to naive catfish under simulated natural conditions.

(7) Successful horizontal transmission of ERV from EUS-affected snakeheads to naive snakeheads under simulated natural conditions.

(8) EUS lesion development in both male and female snakeheads, some of which were gravid.

(9) Apparently healthy snakeheads transmitting EUS rhabdovirus to naive fish thus confirming these as virus carriers.

(10) Virus recovery in tissue culture from naturally exposed experimental fishes.

(11) Virus recovery from several individual organs of naturally EUS-affected and experimentally infected snakeheads demonstrating a lack of organ specificity by the virus.

(12) Demonstration of the influence of temperature on the induction of dermal lesions by virus on naive snakehead juveniles reared at 20 - 22.5 °C but not at ambient water temperature (28 - 32 °C).

(13) The minimum viral dose of > 10^2 $TCID_{50}$ per fish required to induce dermal lesions in naive fish.

(14) Subdermal (SD) viral inoculations as effective in inducing lesions while virus given orally by intubation and by bath did not induce lesions or mortalities.

(15) IP injections of the virus causing mortalities among majority (75 %) of virus-injected fish and 0 % mortality in control fish.

(16) ERV causing significant mortalities ($p<0.01$) in naive snakehead fingerlings with no apparent lesions.

(17) The effect of challenges with various combinations of the rhabdovirus (ERV), the bacterium (*A. hydrophila*) and the fungus, (*Aphanomyces* sp.) on naive snakehead juveniles at low water rearing temperatures (20 - 22.5 °C).

(18) The generation of defective interfering (DI) viral particles by ERV and their consequence on the attenuation of the virus.

The EUS problem is still an annual event given the recent January 1996 monthly mean of 20 % fish morbidity in Laguna de Bay (Chapter 6). Hence, research gaps should be zealously pursued if control of the disease is to be achieved.

Fish farmers should be encouraged to use surviving fishes of EUS epizootics in subsequent breeding of fish for culture. These surviving fishes may be resistant to EUS. Also, stocking of fry or fingerling should not be done during the EUS season when ERV transmission and replication is optimum.

New technologies on disease diagnosis could be used, such as polymerase chain reaction (PCR). Application of this technique will confirm the presence and homogeneity or heterogeneity of ERV in EUS-susceptible fishes during the EUS epizootics in other EUS-affected countries. This technique will also lead to the identification of EUS-susceptible fish species, which are more vulnerable to EUS. PCR will also confirm the exact host range of the ERV as apparently healthy fish appear to be active carriers of the virus. PCR can also be used to delineate areas that are not contaminated by the virus. Information from PCR findings could then be used for a comprehensive plan in EUS prevention.

In addition, this method may be used to detect ERV among other aquatic symbionts such as snails and fish parasites that may serve as vectors of the disease. Initial investigation on tissue filtrates of golden snails from Laguna de Bay yielded positive results (unpublished). Whether the isolated virus is similar to ERV requires further studies. Fish parasites such as *Argulus* sp. are among the numerous parasites associated with EUS fish (Roberts *et al.* 1994). These genera of parasites were reported to be carriers of the spring viremia of carp virus (Pfeil-Putzien 1978; Ahne 1985).

Another research area will be the development of a vaccine using live ERV DI particles. This vaccine would most likely be effectively used in areas where aquaculture of catfish, snakeheads and other economically important fish species that are EUS-susceptible are intensively cultured.

Determination of how the virus initiates infection, thereby completing the current knowledge on the mechanism of pathogenesis of ERV also needs to be investigated. As our studies and that of Kanchanakhan (1996) showed, it appears that viral bath for very

young snakeheads and IM for older fish appear to be the only methods for successfully establishing ERV infections.

Experiments transferring fish artificially infected with virus at low temperatures to high temperature will further confirm persistence of the virus at higher water rearing temperatures. Another experiment involving exposure of naive fish to artificially infected fish will confirm horizontal transmission.

Henceforth, it is recommended that future attempts to isolate ERV should consider sampling either skin lesion or the viscera of grade II lesioned fish, and using a 1:10 dilution of the homogenized fish tissue filtrate for a higher success rate of virus isolation. Collected samples should not be exposed to ambient temperature and should not be refrigerated for too long. Moreover, only CCO or CFS cells should be used for primary isolation and maintenance of ERV.

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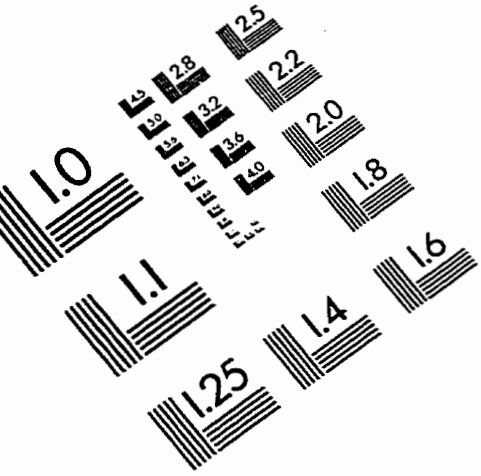
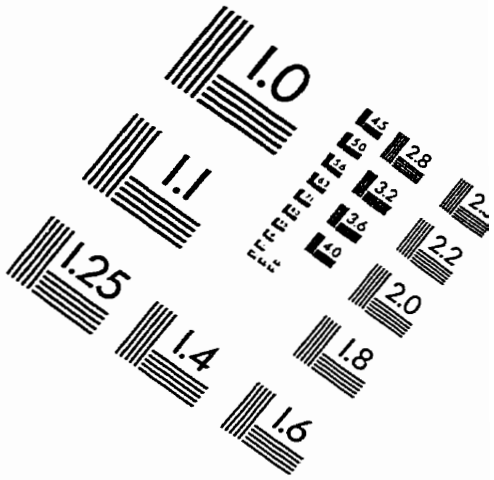
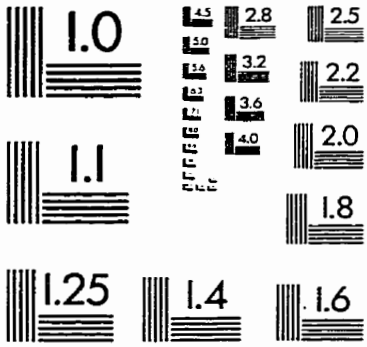
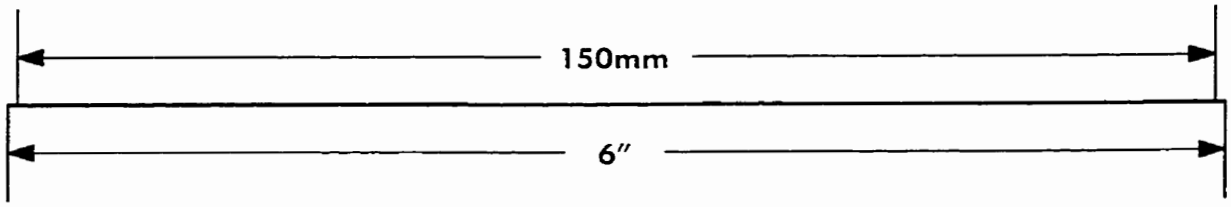
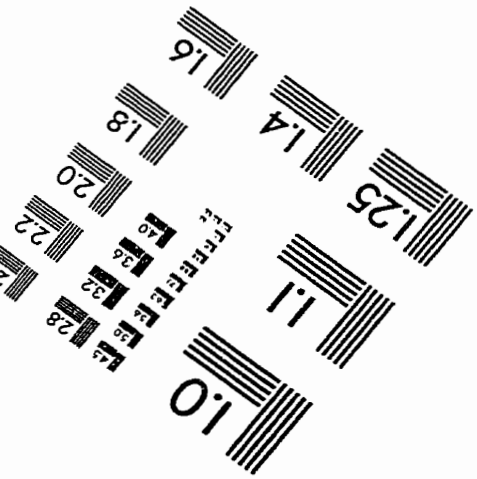
IMAGE EVALUATION TEST TARGET (QA-3)

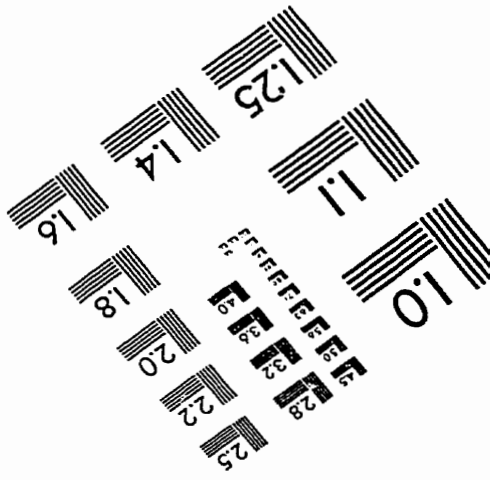












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