Field and Laboratory Studies on Rhabdoviruses Associated with Epizootic Ulcerative Syndrome (EUS) of Fishes

A Dissertation Submitted to the University of Stirling for the Degree of Doctor of Philosophy

by

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Declaration

I declare that this thesis represents the results of research conducted by myself at the Aquatic Animal Health Research Institute, Bangkok, and the Institute of Aquaculture, University of Stirling. The literature consulted has been cited and where appropriate, collaborative assistance has been acknowledged. This work has not been submitted for any other degree.

in memorial of my dad.

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Abstract

Epizootic Ulcerative Syndrome (EUS) is a seasonal and widely spread ulcerative disease condition of fresh and brackishwater fishes in Asia caused by a complex of etiological agents. Viral agents have been found to be associated with EUS but the role of viruses in the complex etiology has still to be identified. Further virological examinations were, therefore, conducted in this study. Two warm-water fish cell lines were established from hybrid catfish, male *Clarias gariepinus* × female *C. macrocephalus*. The HCK line was derived from head kidney and the HCT line was derived from tail tissue. Both lines were susceptible to 3 birnaviruses, sand goby virus (SGV) and infectious pancreatic necrosis virus (IPNV) serotypes Ab and Sp, 2 reoviruses, golden shiner virus (GSV) and catfish reovirus (CRV), but refractory to all 6 strains of ulcerative disease rhabdovirus (UDRV), channel catfish herpesvirus (CCV) and 1 EUS-associated reovirus (T9231). Only the HCK line was susceptible to recent EUS-associated rhabdovirus strain T9204 and tench and chub reoviruses. Using HCK, BF-2 and SSN-1 fish cell lines, 9 rhabdoviruses were successfully recovered from EUS-diseased fishes during the first 2 weeks of a 1993-1994 epizootic. Rhabdovirus strain T9412 caused death in fry and skin damage in juvenile striped snakehead fish. A combination of this rhabdovirus and pathogenic *Aphanomyces* fungus appeared to induce more severe EUS disease in snakehead fish than a single infection with the fungus. EUS transmission was also experimentally achieved by co-habitation of healthy and diseased fish. Three characterised virus strains T9415, T9416 and T9429 possessed a typical bullet- or bacillus-shaped morphology and also exhibited a lyssavirus-like electrophoreotype of structural proteins similar to snakehead rhabdovirus (SHRV) and rhabdovirus strain T9204, while UDRV strains

SL11, BP and 20E possessed vesiculovirus-like eletrophoreotypes. The lyssavirus-like EUS-associated rhabdoviruses, except strain T9416, were structurally and serologically similar for which the 'serotype Sh' is proposed while the UDRV strains are grouped as a proposed 'serotype Ud'. Strain T9416 could not be grouped in either serotype as the homologous antiserum was capable of neutralising viruses of both serotypes. The results of this study suggest that the rhabdovirus is one of a complex of etiological agents for EUS and that at least 2 serotypes of EUS-associated rhabdoviruses are identified.

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Chapter 1

1.1 General Introduction

A new epizootic fish disease syndrome which erupted in South-East Asia in the 1970's has since gradually spread up from the Southeast through to the South of the continent. This syndrome is a seasonal disease condition which usually occurs after heavy rain and during the cooler part of the year and causes ulcerative lesions on the body and/or head of affected fish. Ulcers on the head usually lead to skull erosion and exposure of part of the brain, and loss of eyes and parts of the mouth are frequently found especially in striped snakehead fish, *Channa striatus* Bloch. In many cases, moribund fish have an exposed vertebral column or even complete loss of the posterior part of the body. Most diseased fishes exhibit myonecrosis and fungal granulomas in the muscle tissue (Roberts *et al.*, 1993). The disease has been variously named in different countries. When the disease hit the aquaculture industry in Malaysia and Thailand in the early 1980's it caused very severe losses and drew a great deal of attention from scientists and international organisations. The term 'Epizootic Ulcerative Syndrome' (EUS) was proposed for the disease at the FAO sponsored Expert Consultation on Ulcerative Fish Diseases in the Asia-Pacific Region in Bangkok in 1986 (FAO, 1986) and this designation has been generally accepted by scientists and others concerned with the condition.

The disease condition might first have occured in 1973 in the Mekong River Delta of Vietnam and caused high mortality especially in cultured catfish. EUS recurred and

spread with an unclear pattern to the upper area of the central, to the southwest and to the north of the country and giant snakehead, *Channa micropeltes*, and sand goby, *Oxyeleotis marmoratus*, were also affected (Tuan, 1994). Further outbreaks were identified in over 20 fish species of freshwater and brackishwater fishes in southern Papua New Guinea in 1975-76 where gudgeon fish, *Oxyeleotis* sp., was a major affected species and the similar disease condition appeared in the north in 1982-83 (Tonguthai, 1985). In 1977, Singapore might have been a third EUS-affected country as the information from the Primary Production Department indicated that walking catfish, *Clarias batrachus*, in Sembawang exhibited ulceration on the body without parasitic agents and usually occurred during the dry season (February-April) (Roberts *et al.*, 1986). The ulcerative disease condition first occurred in Java, Indonesia in 1980 affecting common carp, gourami and *Puntius* sp. and spread to other areas in subsequent years from which more affected fishes were recorded such as snakehead fish, catfish, sand goby, jelawat and kissing gouramy (Rukyani, 1994). EUS first occurred in paddy field fishes in many states of Malaysia in 1979-1981 (Lilley *et al.*, 1992) and spread northwards to the southern part of Thailand in late 1981 causing heavy losses of wild and cultured freshwater and brackishwater fishes. The affected fishes developed multiple ulcerative lesions on the body. The epizootic then spread further to most areas of Thailand in 1982-1983 and continued eastwards to Cambodia and Lao P.D.R. and westwards to Myanmar in 1984 (Tonguthai, 1985). Between late 1985 and early 1986, EUS was first reported in Laguna Lake in the Philippines affecting 7 fish species. Within a few years the disease had spread to Naujan Lake in Midoro province and to paddy field and swamp fishes in 11 other provinces of Luzon Island (Bondad-Reantaso *et al.*, 1994). The EUS-diseased estuarine fishes in the Philippines were reported to be usually

associated with acid sulfate soil areas and the outbreak occurred after heavy rainfall (Callinan *et al.*, 1995). EUS was first recognised in Sri Lanka in late 1987, where 30 fish species of fresh, brackish and marine water habitats were reportedly affected of which snakeheads *C. striatus* and *C. punctatus* were the most susceptible fish species (Costa and Wijeyaratne, 1989). In Bangladesh, EUS first occurred in freshwater fishes in the Chandpur district in February 1988 and rapidly spread to all districts of the country (Hossain *et al.*, 1992). Major flooding in September 1988 was believed to hasten the spread in Bangladesh and over 31 fish species were affected (Barua, 1994). EUS moved across the Bangladesh border to India in May 1988 and spread in all directions to affect most areas of the country within the following 3 years involving 7 cultured fish and 23 wild fish species (Das, 1994). Disease outbreaks extended northwards to the subtropical zone of Nepal in February 1989 causing losses of cultured fishes and then spread to the subtemperate zone of the mid-hill area affecting wild fishes in the lake and cultured fishes in ponds and rice fields (Shresta, 1994). Bhutan (in 1989) and southeast China (in 1987) have also reportedly been affected by EUS (Lilley *et al.*, 1992).

Outbreaks of EUS in these 16 countries still recur annually or every few years. High mortality and severely affected fishes have been observed in the early years of the disease in every country. However, severity has decreased in following years. A comprehensive list of affected fishes recorded up to 1992 included more than 100 species (Lilley *et al.*, 1992). Recently in April-May 1996, a similar disease condition first appeared in swamp fishes (*C. punctatus, Labeo rohita, Puntius ticto, P. sophore* and *Cirrhinus reba*) in India's neighboring country, Pakistan. The diseased snakehead fish (*C. punctatus*) were

severely affected and the formalin-fixed specimens were sent to NACA in Bangkok. The ulcerative disease specimens were examined histologically at AAHRI and the EUS condition was confirmed (K. Tonguthai and S. Chinabut, pers. comm.). The outbreak of EUS in Pakistan has immediately suggested that the EUS is still an active spreading epizootic fish disease and may possibly spread to countries of other continents where the predisposing factors meet the requirement of EUS. However, factors required for the development of EUS are still to be identified.

The cause of EUS has been the subject of extensive study since the first outbreak. Agricultural chemicals were initially the major concern as causative factors by scientists in Thailand and Malaysia (Tonguthai, 1985). Paraquat, a herbicide, was believed to be a possible causative factor in Thailand as it was found at 0.030-0.051 ppm in natural water during epizootics (Department of Fisheries, 1983 cited by Menasveta, 1988) and most fish samples were found to be contaminated with this agent (Boonyaratpalin *et al.*, 1983). However, it was later found that the amount of paraquat in diseased fishes was too low to cause any harm to experimental fish (Kanchanopas, 1984; Menasveta *et al.*, 1983; Singhaseni and Tesprateep, 1985). Many agricultural insecticides were also detected in natural water but their concentrations were less than the toxic levels for fish (Tonguthai, 1985). It is, therefore, unlikely that agricultural chemicals were a causative factor.

Within a few years of the first outbreak in Thailand, many workers reported *Aeromonas hydrophila* as the pathogenic bacterium most frequently isolated from the ulcerative lesions of affected fishes with only 1 group of workers isolating *A. sobria* more

frequently than *A. hydrophila*, as reviewed by Tonguthai (1985). Other less frequently isolated bacteria included *Flavobacterium* sp., *Pseudomonas fluorescens*, *Pseudomonas* sp., *Edwardsiella tarda*, *Vibrio parahaemolyticus* and *Streptococcus* sp. (Boonyaratpalin *et al.*, 1983). *A. hydrophila* was also reported as the predominant isolate from affected fishes in different geographical areas such as the Philippines (Llobrera and Gacutan, 1987), Indonesia (Supriyadi, 1988; Karunasagar *et al.*, 1995) and Sri Lanka (Subasinghe *et al.*, 1990). The role of bacteria, especially *A. hydrophila*, as causative agents was unclear since *A. hydrophila* is recognised as a cause of haemorrhagic septicaemia in fish (Roberts, 1993) but was only rarely isolated from internal organs of EUS-diseased fish (Boonyaratpalin, 1989a; Llobrera and Gacutan, 1987; Srisuparp *et al.*, 1984). The isolation of virulent strains of *A. hydrophila* from normal as well as EUS-diseased fish also suggested that this bacterium was only an opportunistic pathogen and not a primary causative agent (Torres *et al.*, 1990). A recent report from Karunasagar *et al.* (1995) also showed that *A. hydrophila* and *A. sobria* could be isolated from diseased and apparently normal fish in the epidemic area and that both bacteria were highly pathogenic to experimentally infected catfish. The authors suggested that *Aeromonas* spp. were secondary pathogens but might contribute to significant mortality in EUS-diseased fishes.

Parasites have also been identified in association with EUS-diseased fishes but were generally disregarded as causative agents and attracted less attention from scientists. A major survey during a 2 year period from 1981-1983 in Thailand found a wide range of parasites in 273 fish specimens which consisted of *Epistylis*, *Chilodonella*, *Trichodina*, *Costia*, *Ichthyophthirius*, *Glossatella*, *Scyphidia*, *Henneguya*, *Myxobolus*, *Thelahanella*,

Dactylogyrus and *Gyrodactylus* (Reungprach *et al.*, 1983). These authors also noted that most of the fish samples had no ulcers on the body. However, *Trichodina* and *Dactylogyrus* were commonly found on snakeskin gourami, *Trichogaster pectoralis*, during the 1982-83 outbreak (Tangtrongpiros *et al.*, 1983a) and *Gyrodactylus*, *Dactylogyrus*, *Epistylis*, *Henneguya* and *Lernaea* were usually found on diseased sand goby, *Oxyeleotris marmoratus*, in cage culture (Supamataya *et al.*, 1983). The common parasites reported in EUS-diseased fishes in India during the first outbreak in 1988 included *Ichthyophthririus*, *Trichodina*, *Epistylis*, *Gyrodactylus* and *Dactylogyrus* (Boonyaratpalin, 1989b).

Fungi belonging the Saprolegniaceae group have been found associated with ulcerative lesions of affected fishes. During the early years of the epizootic, non-septate and un-pigmented fungal hyphae were commonly identified on ulcer surfaces and diseased fish developed chronic mycotic granulomas in the muscle tissue (Chinabut and Limsuwan, 1983). It was noted that these histopathological changes were similar to mycotic granulomatosis in wild fish in Japan described by Miyazaki and Egusa (1973). *Achlya* fungus was isolated from EUS-diseased fish in Thailand (Pichyangkura and Bodhalamik, 1983) and Sri Lanka (Subasinghe *et al.*, 1990). In 1992, *Aphanomyces* was isolated from muscle tissue of EUS-diseased snakehead fish in Thailand and experimentally shown to be pathogenic. Following insertion of *Aphanomyces* hyphae into the muscle tissue of normal fish, the fungus penetrated deep into the body and the fish developed mycotic granulomas similar to naturally diseased fish which was not induced with *Achlya*, *Saprolegnia* or other saprophytic *Aphanomyces* (Roberts *et al.*, 1993). The pathogenic *Aphanomyces* has been rapidly accepted as a major pathogen with a

significant role in the development of EUS lesions (Roberts *et al.*, 1994) and has been named *Aphanomyces invaderis* sp. nov. (Willoughby *et al.*, 1995). However, *A. invaderis* could not immediately invade or infect normal fish following bath challenge (Chinabut *et al.*, 1995; Roberts, 1994) and Chinabut *et al.* (1995) suggested that any viruses or other stressors to which the fish are exposed may induce invasion by pathogenic *Aphanomyces*. It was suggested that this invasive fungus might cause death of EUS-affected fishes and that the fungus was readily eliminated from the fish body by host defense mechanism when the environmental temperature had increased (Chinabut, 1995). Mohan and Shankar (1995) also reported that all diseased specimens collected from fresh or brackishwater during outbreaks in India exhibited fungal infection with a mycotic granulomatous response and it was suggested that the fungus was one of a complex of etiological agents.

A virus has been a long term candidate as a possible causative agent of EUS since the first identification of virus-like particles in diseased fish samples (Rattanaphani *et al.*, 1983; Wattanvijarn *et al.*, 1983a, 1983b, 1984a, 1984b). In 1985, a rhabdovirus and a birnavirus were first reportedly isolated from EUS-diseased fishes in southeast Asia (Wattanavijarn *et al.*, 1986). These reports drew much attention from scientists throughout the region and abroad. Shortly after the first virus isolation, 6 further rhabdovirus strains were recovered from diseased fishes in southeast and south Asia (Frerichs *et al.*, 1986, 1989b) and 2 other birnaviruses from affected fishes in Thailand (Saitanu *et al.*, 1986; Hedrick *et al.*, 1986). Virological studies on EUS-diseased fishes were difficult for all countries in the affected areas as there were no specific laboratories or personnel to undertake fish virology. Information on virological observations was

therefore very limited in some developing countries. In the early 1990's, 3 more viruses were isolated from diseased snakehead fish–1 birnavirus in Singapore (Subramaniam *et al.*, 1993) and 1 rhabdovirus and 1 reovirus-like agent in Thailand (Roberts *et al.*, 1994). The role of a virus as an etiological agent remains uncertain as, firstly, the percentage successful virus isolation from diseased fish has been very low (5%), secondly, no viruses were isolated from many diseased samples from severe epizootics in Bangladesh and thirdly, viruses belonging to 3 different families have been obtained from EUS-diseased fishes (Roberts *et al.*, 1994; Frerichs, 1995). Furthermore, experimental challenge studies on snakehead fish with some virus isolates did not give rise to deep ulcerative lesions similar to naturally diseased fish but only minor skin damage (Frerichs *et al.*, 1993; Saitanu *et al.*, 1986). Thus there has not yet been adequate evidence to indicate a significant role for a virus in the etiology of EUS and viruses might only be present as adventitious agents (Frerichs, 1995). However, the minor skin damage of fish from virus infection experiments may be a predisposing factor for the invasion of *A. invaderis* as suggested by Chinabut *et al.* (1995).

A major international survey was conducted in 1985 covering the EUS affected areas of Thailand, Indonesia, Malaysia, Myanmar, Lao P.D.R., Papua New Guinea and Australia (Roberts *et al.*, 1986). Reports from the survey can be summarised as follows: pesticides were not a causative factor in EUS; no single environmental factor caused the same disease in different geographical areas; outbreaks were likely to occur after heavy rain and in the cool season of the year; there was no direct evidence that bacteria such as *Aeromonas hydrophila* or parasites were primary causative agents; the presence of fungal hyphae in affected fishes from Asia and Australia was not clearly a causative

agent but might cause more severe lesions in affected fishes leading to osmotic imbalance; a rhabdovirus isolated from diseased snakehead fish and swamp eel, *Fluta alba*, might be the primary causative agent (FAO, 1986). Eleven year's research following this international survey has gradually increased understanding of EUS. Scientists have identified 1 specific pathogen, *Aphanomyces invaderis*, but believe that a complex etiology is involved in EUS. In January 1994, the description of EUS was given as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious etiology characterised by the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response" by international agreement at the ODA Regional Seminar on Epizootic Ulcerative Syndrome in Bangkok (ODA, 1994).

Two similar ulcerative fish disease epizootics were also first reported in the early 1970's within the Indo-Pacific region. In 1971 in Japan, an ulcerative disease condition was found in cultured ayu, *Plecoglossus altivelis*, (Egusa and Masuda, 1971). One year later, the outbreak recurred and more fish species were involved. After histopathological examination, the disease condition was named "mycotic granulomatosis" (MG) (Miyazaki and Egusa, 1972). An *Aphanomyces* pathogen was later isolated and named as *Aphanomyces piscicida* (Hatai, 1980). In 1972 in Australia, a similar disease outbreak was reported in mullet, *Mugil cephalus*, in the Bundaberg region of Queensland (McKenzie and Hall, 1976). The disease then spread to different parts of the continent and more fish species were affected. This disease condition was described as "red spot disease" (RSD) (Callinan and Keep, 1989; Fraser *et al.*, 1992). Humphrey and Langdon (1986) considered EUS in Asia to be closely related to RSD in Australia as

both epizootics caused massive death in wild and cultured fishes and outbreaks were widespread. Callinan (1994) suggested that both diseases are the same condition as epizootics are broadly spread, involve many natural and cultured fish species, cause similar clinical signs and share a common pathogenic fungus, *Aphanomyces*. Miyazaki (1994) compared epizootic mycotic granulomatosis in different fish species in Japan. This condition also showed close similarities to EUS and RSD. At the conclusion to an ODA Regional Seminar on Epizootic Ulcerative Syndrome, scientists involved in studies of these 3 epizootics agreed that EUS, RSD and MG are indistinguishable clinical conditions (ODA, 1994). Unfortunately, no virological study was reported from MG disease condition but 1 rhabdovirus was recorded as isolated from diseased fish in Australia in 1985 (Lilley and Frerichs, 1994) and a rhabdovirus-like particle was also found to be associated with RSD (Humphrey and Langdon, 1986; Pearce, 1989). The affected countries with year of first appearance of these 3 similar ulcerative fish diseases are shown in Figure 1.1.

In Thailand, severe losses due to EUS were recorded at nearly US$ 9 million during the second outbreak (Tonguthai, 1985). In Bangladesh, fish losses were estimated at US$ 5.6 million during the first 2 outbreaks (Barua, 1994). Thai fish farmers seem to manage well in preventing EUS by monitoring fish health at the beginning of the cool season, cutting off the water supply when fish show signs of the disease, applying lime at 60-100 kg/rai (1 rai = 1600 m^2) and salt at 200-300 kg/rai when the water becomes poor as recommended by the Department of Fisheries (Tonguthai, 1985).

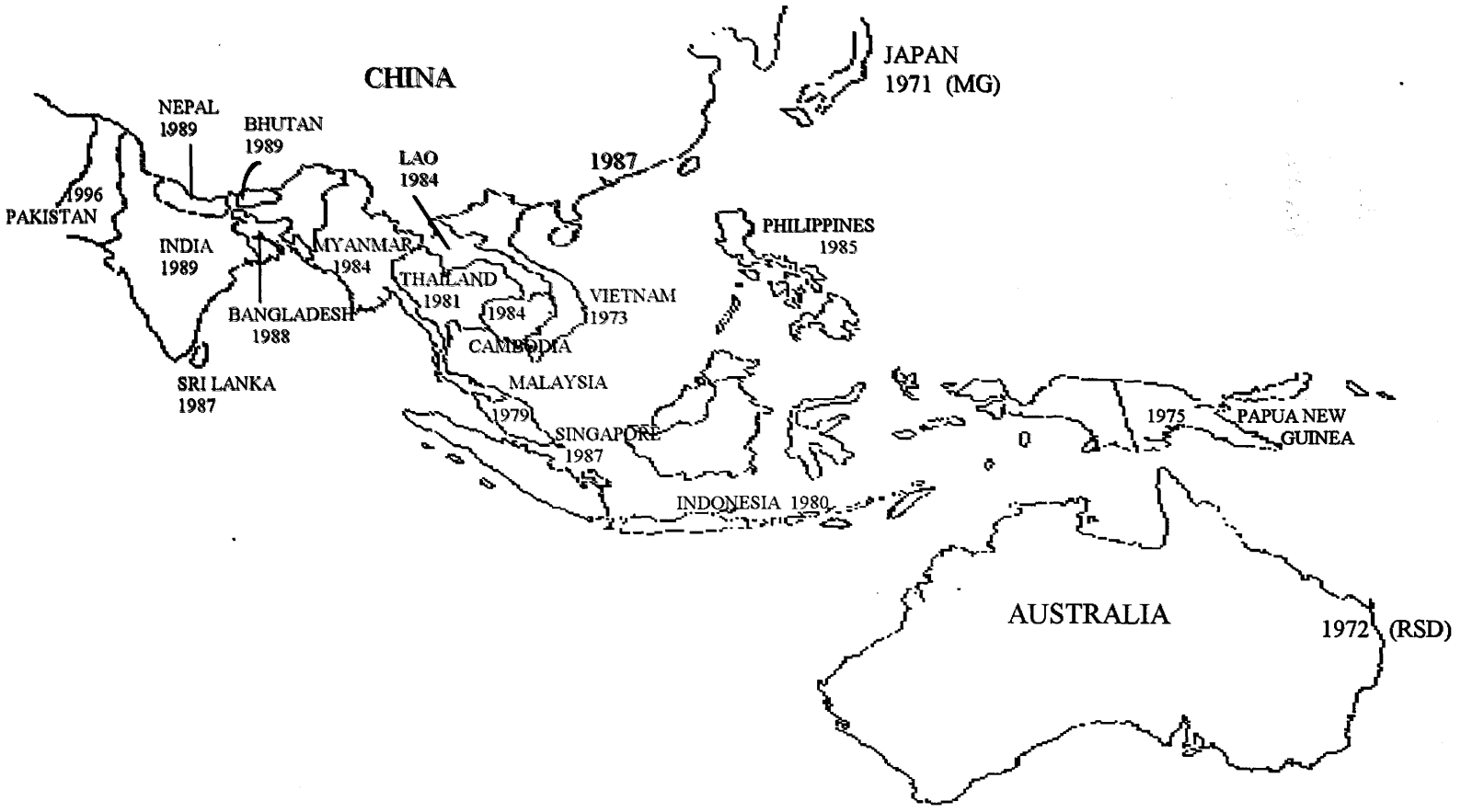


Figure 1.1 Geographical spread and year of first appearance of EUS in Asia-Pacific region. MG and RSD are similar ulcerative disease condition to EUS and are also indicated.

However, fish farmers are still concerned about EUS and fish are generally harvested before the cool season. If the fish are too small to be harvested, the farmer usually keeps stocking density low and reduces the feed during the cool months. Some fish farmers culture fish species such as hybrid catfish (*Clarias gariepinus* × *C. macrocephalus*) during the cool season instead of snakehead fish. Now, more than 2 decades later, the complex of causative agents still to be identified.

1.2 General Objective

The complex of etiological agents causing EUS needs to be identified and the epizootiology of the disease requires further investigation. Previous reports have shown that viral agents are associated with EUS-diseased fishes but how these agents relate to the etiological complex has yet to be determined. Clearly, more virological studies need to be carried out to understand the role of viruses in EUS. It is therefore the general objective of this study to conduct field and laboratory virological studies on epizootic ulcerative syndrome of fishes in southeast Asia with special reference to Thailand.

Chapter 2

Establishment of 2 Hybrid Catfish Cell Lines

2.1 Introduction and Literature Review

World aquaculture has increased rapidly throughout the last decade. To obtain high productivity, the diseases of cultured species need to be understood and controlled. Epizootic ulcerative syndrome (EUS) is a well known epizootic disease of uncertain etiology which has caused massive losses of wild and cultured fishes in many Asian countries. Walking catfish, *Clarias batrachus*, is one cultured species susceptible to EUS and following a few annual epizootics, this fish became a rarity in natural waters and fish farms of Thailand. Broodstock of walking catfish could now take years for recruitment as the disease is still occurring year after year. Most fish farmers have consequently changed to culturing other species because of a short supply of catfish fry and the fear of EUS. During this period, a new culture hybrid catfish was developed in Thailand. This male *Clarias gariepinus* × female *C. macrocephalus* hybrid is one of the most successful cross-bred culture fish with the fast growth characteristic of *C. gariepinus* and good palatability of *C. macrocephalus*. Since the first successful production of the hybrid in 1989 (Nukwan *et al.*, 1990), it has become one of the most economically important species and is now cultured extensively throughout Thailand.

The hybrid catfish is particularly important because fry are exported to many countries in southeast Asia, Bangladesh and India. Although a number of bacterial and parasitic

pathogens have been identified in hybrid catfish farms (Tonguthai *et al.*, 1993), there are no reports of viral disease in this species. Cell lines derived from hybrid catfish should be most suitable for virological examinations of this culture fish and the related genus *Clarias spp.*. Moreover, new cell lines could be used to isolate viruses from EUS diseased fishes.

Studies on fish tissue culture were first described in 1956 by L. Grützner at the Robert Koch Institute in Germany (Wolf, 1988). The first fish cell line was initiated from rainbow trout gonad using a trypsinised tissue technique and mammalian culture media (Wolf *et al.*, 1960). The line was established and named RTG-2 by Wolf and Quimby (1962). Generally, the study of fish cell culture has increased in response to the need for cell substrates to isolate or study fish viruses. A listing up to 1980 showed that 61 fish cell lines had been developed from 36 species belonging to 17 families and that 17 fish viruses had been isolated (Wolf and Mann, 1980). A further 30 new lines were recorded in just 5 reports over a period of only 6 years (Chen and Kou, 1988; Lu *et al.*, 1990; Tung *et al.*, 1991, Fernandez *et al.*, 1993a and Fernandez *et al.*, 1993b). All groups of authors had the primary objective of developing cell lines for fish virology studies and this was well achieved as 29/30 lines were sensitive to many fish viruses. Only one line derived from loach, *Misgunus anguillicaudatus*, was refractory to all 10 tested fish viruses (Chen and Kou, 1988). A recent listing up to 1994 indicated that 159 fish cell lines are now recorded which derived from 74 fish species belonging to 34 families (Fryer and Lannan, 1994).

Fish cell cultures can normally be grown using mammalian culture systems, culture media, serum, antibiotics and buffer solutions without modification except that sea-water fish cell lines may need added sodium chloride in the culture medium to provide isotonic osmolarity (Wolf and Ahne, 1982). The mammalian media most commonly used for fish cell culture are Eagle's minimum essential medium (EMEM), Leibovitz 15 (L-15) and medium 199. A less common medium, modified Ham's F-12, was also used to culture cell lines developed from walking catfish, *Clarias batrachus* (Noga and Hartmann, 1981). Hank's and Earle's salts are widely acceptable buffer systems for the media and pH 7.3-7.4 is optimum for fish cells. Culture media supplemented with 10% serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 25 units/ml nystatin can be used for routine cell culture. Fish cell lines grow well in atmospheric incubators and the culture medium need not to be changed between subculturing (Wolf, 1973).

Identifying cell types in cell cultures based on the morphology of stained or unstained cells has been found difficult in animal cells. Therefore, the terms 'epithelial-like' and 'fibroblast-like' were suggested to describe cell lines unless the cells had a uniform morphology or the origin and/or function of the tissue was known (Schaefer, 1990). However, some lines have showed such differing cell morphology in the same culture flask that they were reported as of mixed epithelial- and fibroblast-like cell types (Noga and Hartmann, 1981; Chen and Kou, 1988; Tung *et al.*, 1991). Garrod (1986) described a complexity of intercellular adhesions in tissues. Epithelial and fibroblast cell types possessed different types and numbers of intercellular adhesions with those of epithelial cells having greater strength than other cell types. Epithelial cells in most animal tissues possess desmosomes, a type of intercellular adhesion composed of two symmetrical half-

desmosomes (hemidesmosomes) on adjacent cells that form a link called the 'intercellular mid-line' between 2 cells, except in the pigmented layer of the eye and the epithelial cells of the lens. Only cardiac muscle of the non-epithelial cells possesses desmosomes. However, using desmosomes as an epithelial cell type indicator has not yet been universally adopted for cell cultures.

Chromosome characterisation for cell identity is required for certified cell lines in the American Type Culture Collection (ATCC) (Hay, 1985) as cross contamination found between higher vertebrate cell lines used in medical research gave rise to much misunderstanding or uncertain results because each cell line was sensitive to different viruses (Nelson-Rees *et al.*, 1981; Nelson-Rees, 1983). These authors suggested karyotyping for the identification of new cell lines should be introduced into the laboratory. However, there have been no reports of similar cross contamination in lower vertebrate cell lines.

Mycoplasma contamination in animal cell cultures is difficult to avoid as the usual sources are people, serum used for cell cultures, and tissue of donor animals. However, primary cell cultures show less mycoplasma contamination than continuous cell lines, and a contaminated cell line can be a major source of mycoplasmas spreading to other cell lines in the laboratory (McGarrity *et al.*, 1985). It was initially believed that tissue from donor fish had never been a source of mycoplasma (Wolf and Ahne, 1982) but since the report of mycoplasma isolation from tench (*Tinca tinca* L.) by Kirchhoff *et al.* (1983), a fish donor might be an additional source of mycoplasma contamination in the fish cell culture laboratory. Mycoplasmas can degrade host cells and cause changes in

host cell DNA (McGarrity, 1982). Therefore, newly established cell lines need to be tested for mycoplasma contamination. The many methods for the detection of mycoplasma in cell culture are well documented in *Diagnostic Mycoplasmology* (Tully and Rasin, 1983).

Fish cell culturists have established fish cell lines with the similar characterisation required for Certified Cell Lines (Hay, 1985). The characteristics normally used are cell viability before freezing and after thawing; cell growth; plating efficiency; stained and unstained cell appearance; karyology; micro-organism contamination; adventitious viruses; virus susceptibility. Fish cell lines have been used in many fields of research including toxicology, genetics, cell biology and physiology, oncology and immunology as documented by Nicholson (1989), Bols (1991), Bols and Lee (1991) and Babich and Borenfreund (1991). However, the purpose of the present work was to establish 2 hybrid catfish cell lines for future fish virological studies, especially those viruses associated with EUS.

2.2 Objective:

-To establish and characterise 2 hybrid catfish cell lines

2.3 Materials and Methods

Primary Cell Preparation

The first cell line, HCK (hybrid catfish kidney), was derived from the head kidney of a hybrid catfish (male *Clarias gariepinus* × female *C. marcrocephalus*), 20 cm in total length. A primary cell culture was prepared using the minced tissue technique similar to Wolf and Quimby (1976a). Briefly, the donor fish was anaesthetised with an over-dose of quinaldine and the abdomen aseptically opened. Portions of the head kidney were removed, placed in a sterile petri dish and then antibiotic solution (1000 units penicillin, 1000 µg streptomycin, 5 µg amphotericin B and 250 µg gentamicin per ml EMEM) (Sigma, USA) was added just to cover the tissue to eliminate microbial contamination. Tissue was minced into 1-2 mm^3 pieces using 2 sterile razor blades and then transferred to two 25 cm^2 tissue culture flasks (Nunc, Denmark). Most of the liquid was drained off and the tiny pieces of tissue were allowed to adhere to the flasks for about 1 h before adding 3-4 ml medium M-199 (Seromed, Germany) supplemented with 20% fetal calf serum (FCS) (Seromed, Germany) and 2× antibiotics (200 units penicillin and 200 µg streptomycin per ml). The primary cell cultures were incubated at 28°C. This culture medium formulation was used for the first 3 subcultures.

The second cell line, HCT, was derived from the tail of a hybrid catfish 14 cm in total length. A primary cell culture was prepared using the trypsinised tissue technique of Wolf and Quimby (1976b) with minor modifications. The fish was sacrificed and the tail removed and placed in 2000 ppm hypochlorite solution for 5 min. The tail was then washed with running tap water and 3 litres of sterile distilled water, cut into small pieces and transferred to an Erlenmeyer flask. Phosphate buffer saline (PBS) containing antibiotics (250 units penicillin, 250 µg streptomycin and 250 µg kanamycin per ml) was

added and the tissue incubated for 60-90 min at 25°C. The PBS was drained off and fresh PBS with antibiotics and 0.25% trypsin was added to the flask. The small pieces of tissue were stirred at room temperature using a magnetic stirrer. Every 30 min over a 2 h period, the dissociated cells were transferred to a centrifuge tube and excess trypsin neutralised by adding a few drops of serum per 10 ml of cell suspension. Fresh PBS with antibiotics and trypsin was added to the tissue in the Erlenmeyer flask and the trypsinisation process continued. Harvested cells were collected by centrifuging at 1500 rpm (280×g) for 5 min (Denley BR401 refrigerated centrifuge, UK) and washed twice with PBS. The number of viable cells was determined by staining with trypan blue and counting in a haemocytometer as described by Freshney (1987). The cells were diluted with culture medium to obtain 10^5-10^6 cells/ml. One ml of cell suspension was seeded into a 25 cm^2 tissue culture flask (Nunc, Denmark) and 7 ml of EMEM (with 20% FCS, 2 mM L-glutamine and 2× antibiotics) added before incubation at 28°C. This culture medium was used for the cell line between passages 1-3.

After passage 3, medium L-15 supplemented with 10% serum and 1× antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) or EMEM supplemented with 10% serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1× antibiotics were used as routine culture media for both cell lines.

Cell Line Subculture

A procedure similar to that described by Wolf and Quimby (1976c) was used for cell line subculture. Culture medium was discarded from confluent monolayers and the cell sheet washed once with 5-7 ml PBS. The PBS wash was removed and replaced with 1 ml

trypsin-EDTA solution (Flow, UK) per 25 cm^2 flask. The flask was gently tilted to ensure trypsin-EDTA covered all the cell sheet surface and left for a few minutes in the tissue culture cabinet. When the cell sheet turned opaque, the trypsin-EDTA was removed and the cell sheet loosened by gently tapping the flask with the palm of the hand. For subculturing at 1:3 ratio, 6 ml of culture medium was added to the flask and the cells triturated with a 10 ml pipette to obtain a single cell suspension. Two ml of cell suspension was seeded into each of 3 new 25 cm^2 flasks containing 6 ml of culture medium. Cultures of HCK and HCT cells were routinely incubated at 28°C.

Viable Cell Count

Single cell suspensions obtained after trypsinisation were counted using a dye exclusion method and haemocytometer. Briefly, 100 µl of single cell suspension and 100 µl of trypan blue staining solution (Flow, UK) were mixed in an eppendorf tube. Cells were counted in a haemocytometer at ×200 magnification. Live cells do not absorb the dye, while dead cells are stained blue. The method, counting technique and calculation were as described by Freshney (1987).

2.3.1 Cell Morphology

The hybrid catfish cell lines cultured as monolayers in 25 cm^2 tissue culture flasks were examined for unstained appearance by phase contrast under an Olympus IMT-2 inverted microscope. For observation of stained cell morphology, the cell lines were cultured in flaskettes (Nunc, Denmark). The cell sheets were fixed in methanol for 10 min, stained with 0.25% May-Grunwald for 10 min and double stained with Giemsa in buffer pH 6.8

for 10 min (Adams, 1980). The stained cells were examined under normal light microscopy. For ultrastructure studies, confluent cell sheets in 25 cm^2 flasks were fixed with Karnovsky's fixative (Karnovsky, 1965) and detached using a cell scraper. Fixed cell suspensions were transferred to centrifuge tubes and spun at 2000 rpm (580×g) for 10 min at 4°C to obtain a cell pellet. Fixative was changed and the pellet cut into small pieces of 1-2 mm^3. The pieces were allowed to fix completely in the centrifuge tube for further 15-30 min at 4°C and were then transferred to a sample bottle with fresh fixative and held for another 2-4 h at 4°C. Cell pellets were rinsed 2-3 times and held overnight in cacodylate buffer solution at 4°C. The cacodylate buffer was removed and 0.5% osmic acid in cacodylate buffer added and left for 1 h. The cell pellets were dehydrated, embedded in epoxy resin (Araldite CY212), and cut with an ultramicrotome according to a general tissue preparation method for transmission electron microscopy (Hayat, 1989). Thin sections were placed on EM grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) solution. Samples were viewed under a Philips 301 transmission electron microscope at 80 kV.

2.3.2 Growth at Different Temperatures

Growth of HCK passage 60 and HCT passage 15 in EMEM medium supplemented with 10% FCS (EMEM-10) at different temperatures was determined. The experiments were conducted in 25 cm^2 flasks with 2 replications. Cells were trypsinised and counted then diluted and suspended in EMEM-10. Single cell suspensions of HCK and HCT lines at 5.8×10^5 and 5.5×10^5 cells/ml, respectively, were prepared. Aliquots of 1 ml of HCK cells were seeded into each of 8 tissue culture flasks and 1 ml of HCT cells seeded into a

further 8 flasks. A 7 ml quantity of EMEM-10 was added to every flask. Two sets of flasks of each line were incubated at 15, 22, 28, or 37°C. Cell growth was determined by daily visual assessment of confluency of the monolayers on the flask surfaces.

2.3.3 Growth at Different Serum Concentrations

Growth of HCK passage 64 and HCT passage 22 cells in EMEM medium supplemented with different serum concentrations was determined. The experiments were conducted in 25 cm^2 flasks with 2 replicates. Cells were counted as previously described, then diluted and suspended in serum free EMEM medium. Single cell suspensions of HCK and HCT lines contained 3.4×10^5 and 4.0×10^5 cells/ml, respectively. Aliquots of 1 ml of HCK cells were seeded into each of 10 tissue culture flasks and 1 ml of HCT cells was seeded into another 8 flasks. Two sets of flasks of each line received 7 ml of EMEM containing 2, 5, 10 or 20% FCS. An additional 2 flasks of HCK cells were cultured in serum-free EMEM. Cell growth was determined by daily visual assessment of confluency of the monolayer on the flask surface.

2.3.4 Cryopreservation

HCK and HCT cells were cultured in antibiotic-free EMEM-10 for 3 passages before cryopreservation. Cultures of HCK passage 55 and HCT passage 18 were trypsinized, counted and diluted to ~10^6 cells/ml in antibiotic-free EMEM-10 with 10% dimethyl sulfoxide as cryopreservative. A 1 ml volume of each cell suspension was distributed in 18 cryotubes for the HCK line and 14 cryotubes for the HCT line. Cells were kept at

4°C for 30 min then transferred overnight to a deep freezer at -70°C. On the following day, 6 tubes of HCK and 4 tubes of HCT cells were transferred to liquid nitrogen and the balance retained at -70°C. After 24 h storage, tubes from each temperature were thawed in warm water for 30-60 sec and cell viability determined as previously described. Cells were seeded into 25 cm^2 tissue culture flasks and 10 ml EMEM-10 added to each flask. Cultures were incubated at 28°C overnight and the medium replaced with fresh EMEM-10 the next day. The number of days to achieve monolayer confluency was recorded.

2.3.5 Plating Efficiency

A test of the ability of single cells to form colonies was conducted in 25 cm^2 flasks according to Fryer *et al.* (1981). Single cell suspensions of HCK passage 60 and HCT passage 22 were counted and diluted to 10^4, 10^3 and 10^2 cells/ml. One ml aliquots were seeded into flasks with 3 replicates for each cell line and incubated at 28°C. Medium EMEM-10 was used for growth. The cultures were observed for 14 days. The experiments were terminated when single cells had grown to small colonies (4-6 cells/colony). The cells were fixed in formalin fixative (25% formalin, 10% ethanol and 0.5% acetic acid) for 5 min and stained with 1% crystal violet in formalin fixative for 30 min (Fryer *et al.*, 1981). The colonies were counted under an inverted microscope. The percent efficiency of plating was calculated as follows:

$$\text{Plating efficiency (\%)} = \frac{\text{No. colonies formed} \times 100}{\text{No. cells seeded}}$$

2.3.6 Microorganism Contamination

Both cell lines were thawed from liquid nitrogen and cultured in antibiotic-free medium for at least 3 passages. Cells showing 80% confluency were scraped off and triturated using a cell scraper (Nunc, Denmark) and 10 ml pipette. One ml of each cell suspension was inoculated into tubes containing 10 ml trypticase soy broth, 10 ml Sabouraud dextrose broth or 10 ml thioglycollate broth media prepared according to Hay (1985). Four replicate tubes were used for each cell line with 2 tubes incubated at 22°C and the other 2 tubes at 37°C. Tubes were observed for 14 days for turbidity caused by bacteria or fungus growth compared to un-inoculated control tubes.

2.3.7 Mycoplasma Contamination

Mycoplasma isolation and DNA staining using Hoechst stain were used for the detection of mycoplasma contamination. Details of these tests are given by Adams (1980), McGarrity (1982) and McGarrity *et al.* (1985).

For mycoplasma isolation, cell suspensions of HCK passage 60 and HCT passage 20 were prepared as described previously and 100 µl of suspension was dropped and spread on triplicate mycoplasma agar plates. A mycoplasma-contaminated snakehead cell line, CCSN, (Institute of Aquaculture, un-established cell line) was used as a positive control. The plates were sealed with flexible plastic film sheets (Nescofilm, Japan) and incubated aerobically at 37°C. To increase the sensitivity of the isolation procedure, 1 ml of cell suspension was also first inoculated into mycoplasma broth and incubated for 3 days at

37°C and 100 µl broth then transferred onto mycoplasma agar plates. The plates were observed for 2-3 weeks for the appearance of fried egg-like mycoplasma colonies.

For DNA staining of mycoplasma, the commercially available Mycoplasma Stain Kit (ICN Biomedicals, UK) was used. Cells were cultured in flaskettes and when growth covered 70-80% of the surface area of the glass slides, the cells were fixed with Carnoy's fixative (acetic acid : methanol = 1 : 3) for 15 min with the fixative changed once after 5 min. Cells were dried and stained with Hoechst stain solution for 30 min. Positive and negative mycoplasma control slides supplied with the kit were also stained. The slides were rinsed with de-ionised distilled water and covered with a coverslip using the mounting reagent supplied. Light at 360 nm produced optimum for excitation of Hoechst-stained DNA and was emission at 490-500 nm. Cells were observed for tiny illuminated dots of mycoplasma nuclei in the cell cytoplasm at ×400 under fluorescence microscopy (Leitz, Germany) with an exciter filter UG1-UV and barrier filter BG-38.

2.3.8 Chromosome Analysis

HCK passages 19 and 63 and HCT passage 20 cells were cultured in two 80 cm^2 flasks incubated at 28°C. When the cells reached 80% confluency, the culture medium was replaced with fresh medium containing 0.5 µg/ml colcemid. The cells were incubated for a further 4 h and harvested using cold trypsin solution. Harvests were transferred to a centrifuge tube and 5 volumes of hypotonic solution (EMEM-10 : distilled water = 1:2) were added. The treated cells were incubated at room temperature for 10 min and collected by centrifugation at 1000 rpm (160×g) at 4°C for 5 min. The cells were fixed

by adding 4 volumes of ice-cold Carnoy's fixative and holding at 4°C for 20 min with periodic gentle shaking. The cells were spun and re-suspended twice in 3 ml fixative. Fixed cells were delivered drop-wise on to clean slides from a height of 80 cm, dried quickly and stained with 2% Giemsa in 0.5 M PBS at pH 6.8 for 10 min. Chromosome spreads from 100 cells were counted under 1000× magnification. This method was modified from Adams (1980) and Alvarez *et al.* (1991).

2.3.9 Adventitious Viruses

The examination for adventitious viruses was conducted using two 24-well plates. Each plate was marked off into 4 sections of 6 wells and each section seeded with epithelioma papulosum cyprini (EPC) (Fijan *et al.*, 1983), fathead minow (FHM) (Gravell and Malsberger, 1965), brown bullhead (BB) (Wolf and Quimby, 1969) or striped snakehead (SSN-1) (Institute of Aquaculture, un-established cell line) indicator cell lines. HCK and HCT cells were detached and dispersed using cell scrapers and 10 ml pipettes and 100 µl volumes of cell suspension inoculated onto each of 4 wells of each indicator cell line. The remaining 2 wells of each line were inoculated with 100 µl of L-15 medium supplemented with 2% serum as negative controls. The hybrid catfish cells were allowed to adsorb for 45 min before 1.5 ml of L-15 medium supplemented with 2% serum was added to each well. The plates were sealed and incubated at 25°C and observed daily for 14 days for cytopathic effects (CPE). One passage of culture fluids on to new indicator cell cultures was carried out on day 8-10 post-inoculation.

2.3.10 Susceptibility to Virus Infections

Sixteen fish virus strains were tested for infectivity in the HCK and HCT cell lines. These were comprised of 3 birnaviruses, 5 reoviruses, 1 herpesvirus and 7 EUS-associated rhabdovirus isolates (Table 2.1). Each virus was propagated in a selected permissive cell line. When the cells showed complete CPE, they were spun at 2500 rpm (800×g) for 20 min and the virus containing supernatant fluid was collected. The amount of virus was determined by a quantal assay in cell culture as described by Burleson *et al.* (1992). The 50% tissue culture infective dose ($TCID_{50}$) was calculated according to the Spearman-Kärber method (Kärber, 1931) (Appendix 1). Susceptibility to virus infection of HCK and HCT lines was carried out in 24-well plates containing 80% confluent monolayers. Culture medium was removed and 100 µl undiluted virus stock inoculated on to each of 4 replicate wells. Four wells remained as negative controls for each plate. After 45 min adsorption, 1.5 ml of EMEM medium supplemented with 2% serum was added to each well. The cells were incubated in 2% CO_2 at 20°C and observed daily for CPE (Note: Two CO_2 incubators were available in the laboratory–one was set at 15°C and the other was set at 20°C). On day 10, a 200 µl sample of supernatant from each well was subcultured to new preformed monolayers in 24-well plates. The plates were observed for a further 14 days.

Table 2.1 Fish viruses selected to test the virus susceptibility of HCK and HCT cell lines.

Virus	Designation	Source	Reference
Rhabdoviruses			
EUS-associated viruses	T9204	Institute of Aquaculture	Lilley and
	20E	University of Stirling, UK	Frerichs, 1994
	O2		
	SL11		
	BP		
	19		
	A4		
Birnaviruses			
Infectious pancreatic necrosis virus	IPNV serotype Sp and Ab	Institute of Aquaculture, UK reference strains	
Sand goby virus	SGV	Prof. JL Fryer, OR, USA	Hedrick *et al.*, 1986
Herpesvirus			
Channel catfish virus	CCV	Dr. P Dixon, Weymouth, UK	Fijan *et al.*, 1970
Reoviruses			
Golden shiner virus	GSV	Prof. RP Hedrick, CA, USA	Plumb *et al.*, 1979
Channel catfish reovirus	CRV	Prof. RP Hedrick, CA, USA	Hedrick *et al.*, 1984
Tench reovirus	Tench	Prof. W Ahne, Germany	Ahne and Kölbl, 1988
Chub reovirus	Chub	Prof. W Ahne, Germany	Ahne and Kölbl, 1988
EUS-associated reovirus	T9231	Institute of Aquaculture, UK	Roberts *et al.*, 1994

2.4 Results

These are the first 2 established cell lines derived from hybrid catfish (*Clarias gariepinus* × *C. macrocephalus*).

Minced head kidney tissue adhered to the culture flask surface within 5 min of seeding and started to spread out from pieces of tissue. For the very small pieces, cells spread out evenly within 5 days as focal cell sheets. For the larger pieces, some tissue remained at the center of focal cell sheets. Cells at the edges were larger than cells towards the center of the sheets. Cells showed contact inhibition and grew to cover the whole surface of the culture flask by day 14, with retained pieces of tissue adhering to the flask surface. After a few passages, cells from these pieces of tissue spread out entirely and an even monolayer was obtained. This head kidney-derived cell line was designated HCK (hybrid catfish kidney). HCK grew well in M-199, EMEM and L-15 media supplemented with FCS. The cell line achieved confluency within 3 days at a split ratio of 1:3.

The second cell line was derived from a tail of hybrid catfish and designated HCT (hybrid catfish tail). The trypsinised tissue technique provided a high density viable cell suspension. Cells were seeded into 25 cm^2 tissue culture flasks at 10^5-10^6 cells/flask and L-15 (20% FCS, 2× antibiotics) was used as culture medium. Most cells adhered to the surface by day 5 after seeding. Half of the L-15 medium was changed twice to remove unattached cells and debris and cell monolayers were obtained by day 9-10 post-seeding. The first subculture was performed on day 14. A split ratio of 1:2 was suitable for HCT

and the monolayers took 5-6 days to achieve confluency. HCT cells grew well in L-15 and EMEM media supplemented with FCS.

2.4.1 Cell Morphology

HCK and HCT at low passage levels showed a mixed epithelial-like and fibroblast-like cell type morphology. Both cell lines developed a more uniform cell type at higher passage levels (Figures 2.1-2.2). Most HCK and HCT cells adhered to the flask surface within a few minutes after seeding to new flasks. Cells showed mitotic division with the clear appearance of metaphase, anaphase and telophase stages in HCK cells. When cells reached metaphase stage, the center of the cell became enlarged and raised because of the alignment of homologous chromosomes. Cells started to elongate and the homologous chromosomes separated and moved towards both ends of the cell in the anaphase stage. Two daughter cells were obtained in the telophase stage. Desmosome adhesions were identified in an electron micrograph of HCT passage 18 cells (Figure 2.3a). A clear micrograph of desmosomes is shown in Figure 2.3b in which the tissue sample was prepared from a cluster of HCT cells. Similar intercellular adhesions were not observed in HCK passage 56. These findings indicate that HCT is an epithelial cell type while HCK is a fibroblast-like cell type.

2.4.2 Growth at Different Temperatures

HCK passage 60 cells achieved confluency within 5, 3 and 2 days at 22, 28 and 37°C incubation, respectively, after seeding at 5.8×10^5 cells/flask. At 37°C incubation, HCK

Figure 2.1 Cell appearance of HCK and HCT.

a. Unstained HCK passage 55. Bar = 90 μm

b. Unstained HCT passage 36. Bar = 90 μm

c. Giemsa-stained HCK passage 66. Bar = 60 μm

d. Giemsa-stained HCT passage 21. Bar = 60 μm

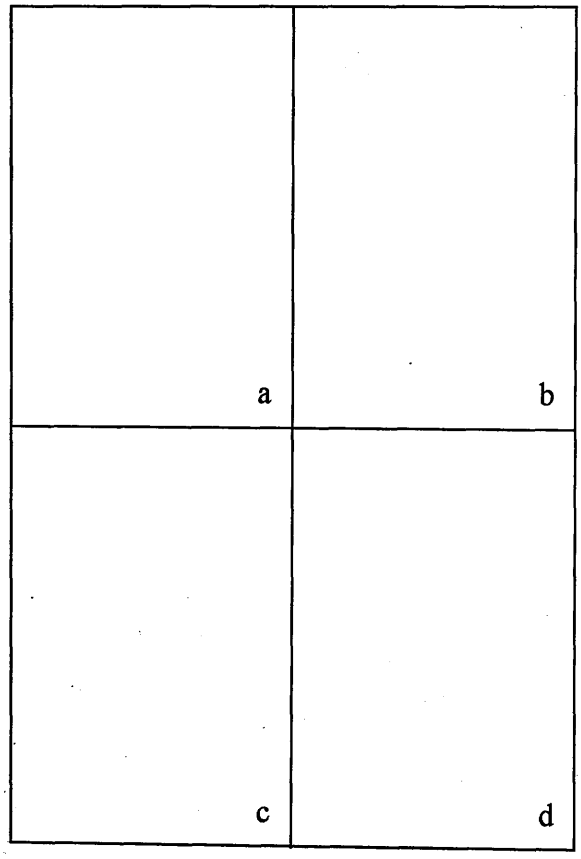
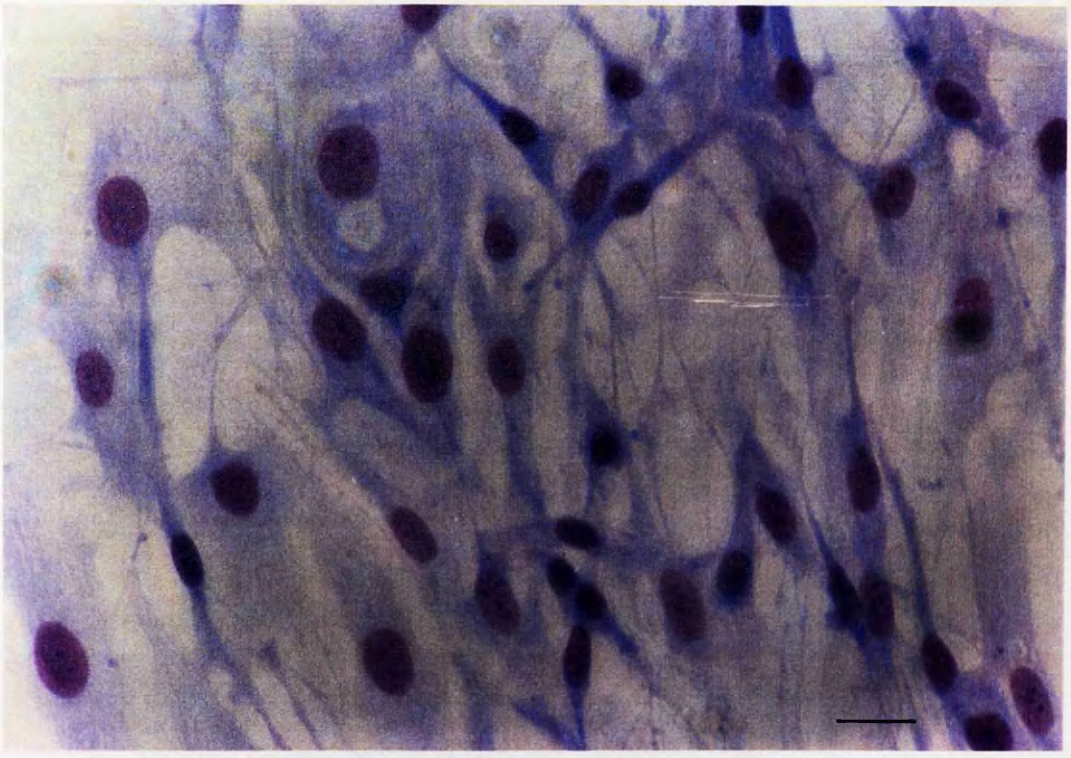


Figure 2.2 Giemsa-stained HCK and HCT (colour print).

a. HCK passage 66. Bar = 13.3 μm

b. HCT passage 21. Bar = 13.3 μm



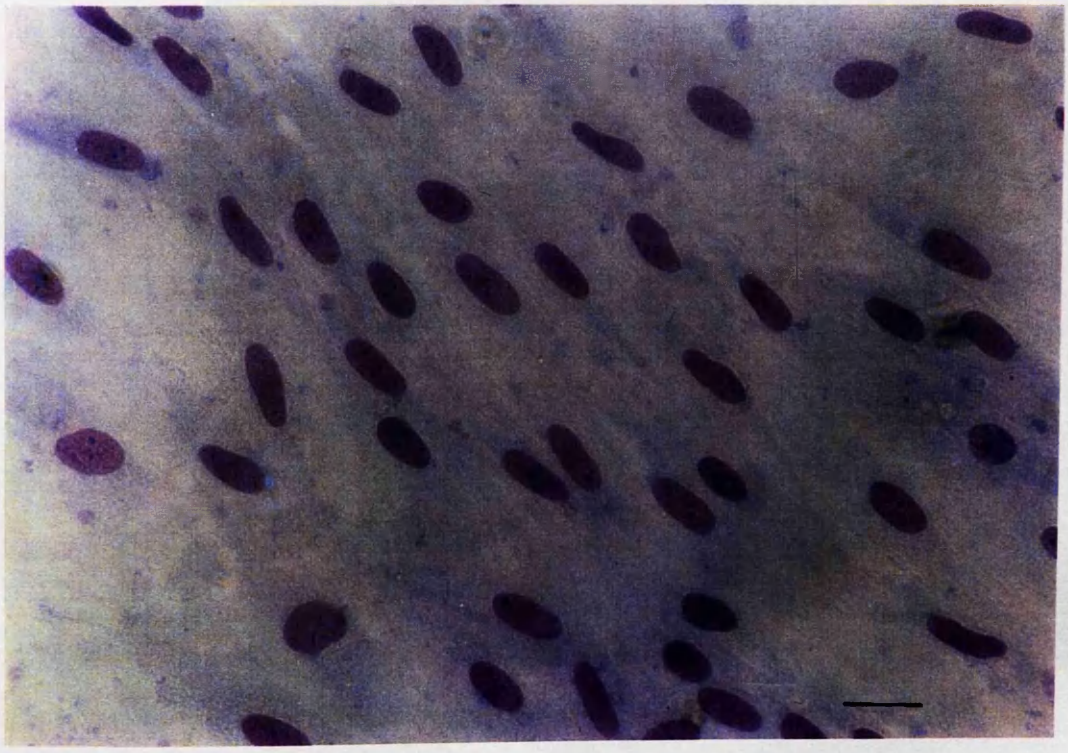
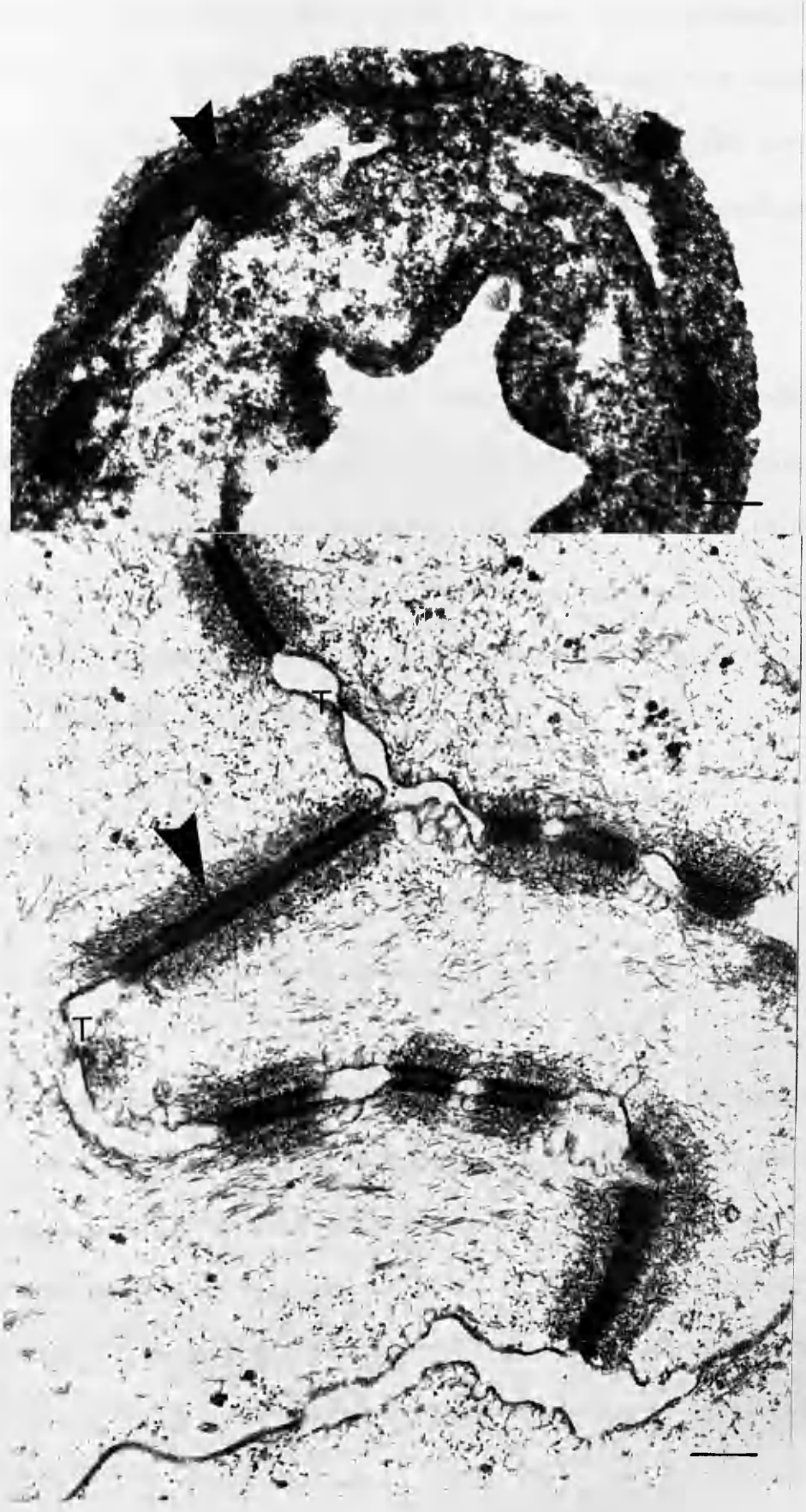


Figure 2.3 Electron micrograph of HCT cells showing intercellular adhesions. Desmosomes are cell-cell adhesions indicating epithelial cell type. Tight junction is another type of cell-cell adhesion which helps to reduce intercellular space (Garrod, 1986). Desmosomes were not found in HCK cells.

a. Desmosome adhesion (arrow) of HCT passage 18. Sample prepared from cell monolayer. Bar = 180 nm

b. Desmosome adhesion (arrow) and tight junction (T) of HCT cells at unknown passage number. Sample prepared from cluster of cells. Bar = 190 nm


T
T

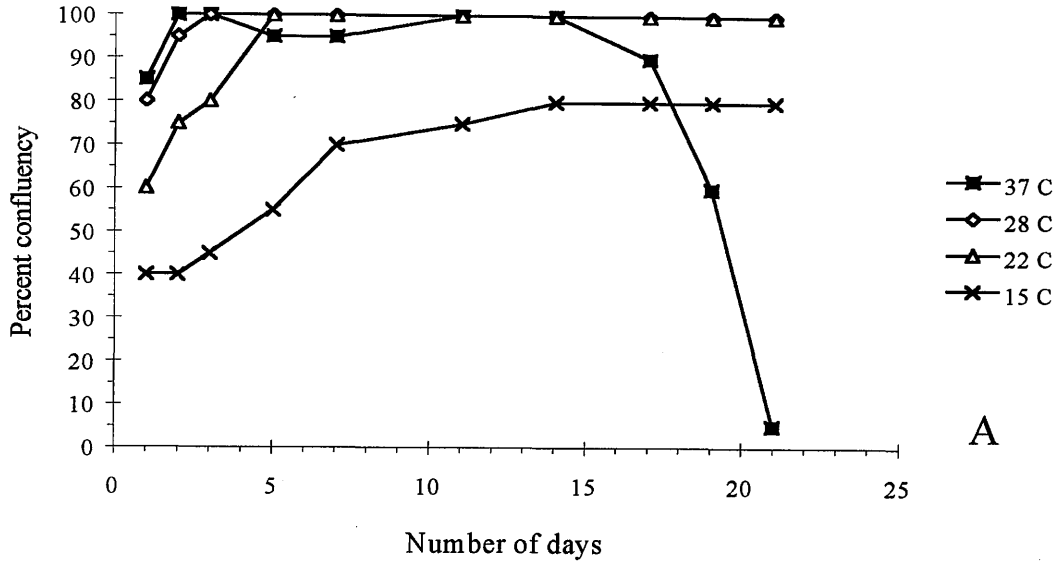
grew rapidly during the first few days followed by a period of cell degeneration lasting for 7 days before the cells recovered, grew, and achieved confluency for the second time lasting for a few days (Figure 2.4a). At 15°C incubation, HCK grew very slowly and never reached full confluency. HCK cells at 22 and 28°C remained fully confluent until days 47 and 28, respectively (Figure 2.4b).

HCT passage 15 cells achieved confluency within 3 and 2 days at 22 and 28°C incubation, respectively, after seeding at 5.5×10^5 cells/flask. At 15°C incubation, HCT grew slowly and reached only 70% confluency at day 8 and remained so until day 25 before starting to degenerate. Although HCT cells grew during the first week at 37°C incubation, they degenerated rapidly after day 8. HCT cells at 22 and 28°C remained fully confluent until days 41 and 29, respectively (Figure 2.5a-b).

Both cell lines grew best at 22 and 28°C.

2.4.3 Growth at Different Serum Concentrations

HCK cells at passage 64 were able to grow to full confluency by days 11, 8, 6 and 6 in 2, 5, 10 and 20% FCS containing media, respectively, after seeding at 3.4×10^5 cells/flask. There was no growth in serum-free EMEM medium. At a 2% serum level, the growth of HCK cells slowly reached confluency then rapidly degenerated. The growth of HCK cells cultured in 5, 10 and 20% serum containing media was similar and confluent cell sheets remained intact over 2 months without replacement of medium (Figure 2.6a-b).



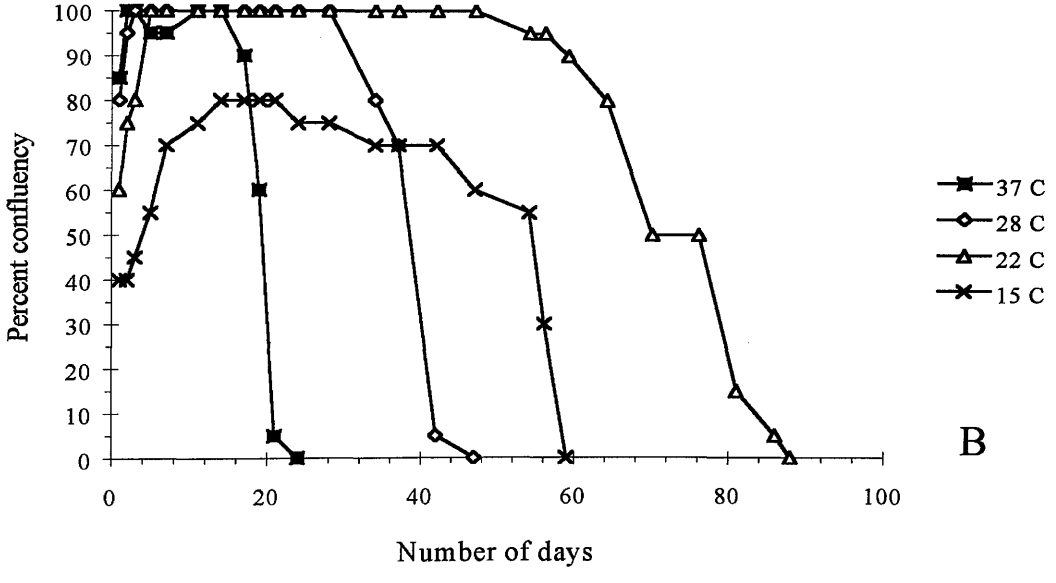
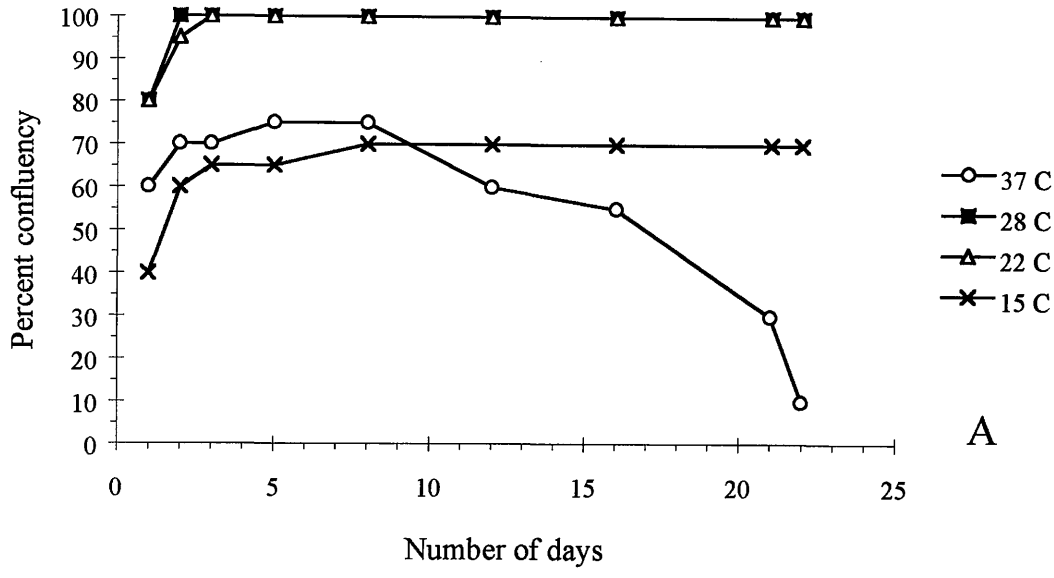


Figure 2.4 Growth of HCK passage 60 cultured in EMEM supplemented with 10% FCS and incubated at 4 different temperatures. HCK seeded at 5.8×10^5 cells/flask. (A) Growth up to 21 days. (B). Growth up to 88 days.



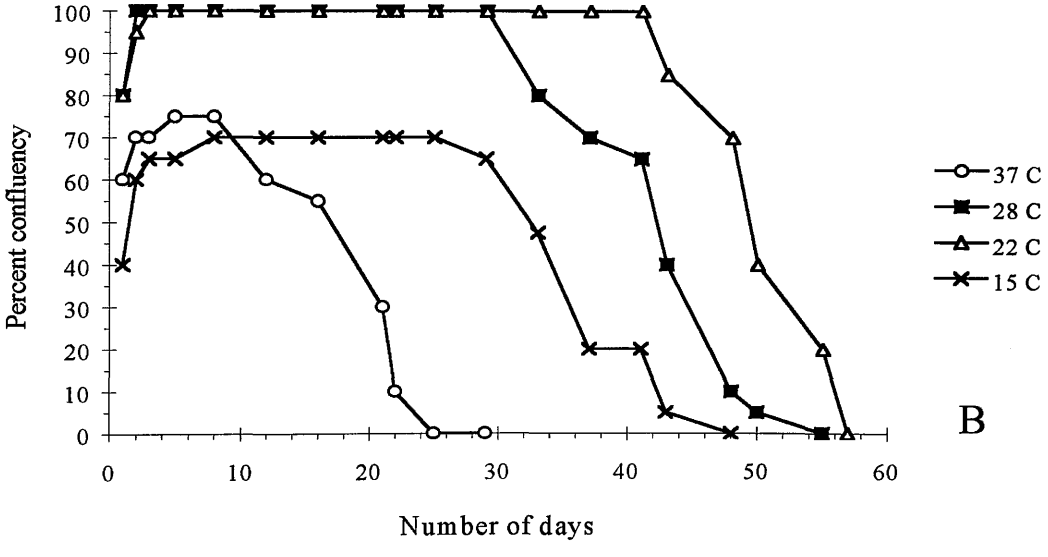


Figure 2.5 Growth of HCT passage 15 cultured in EMEM supplemented with 10% FCS and incubated at 4 different temperatures. HCT seeded at 5.5×10^5 cells/flask. (A) Growth up to 22 days. (B) Growth up to 57 days.

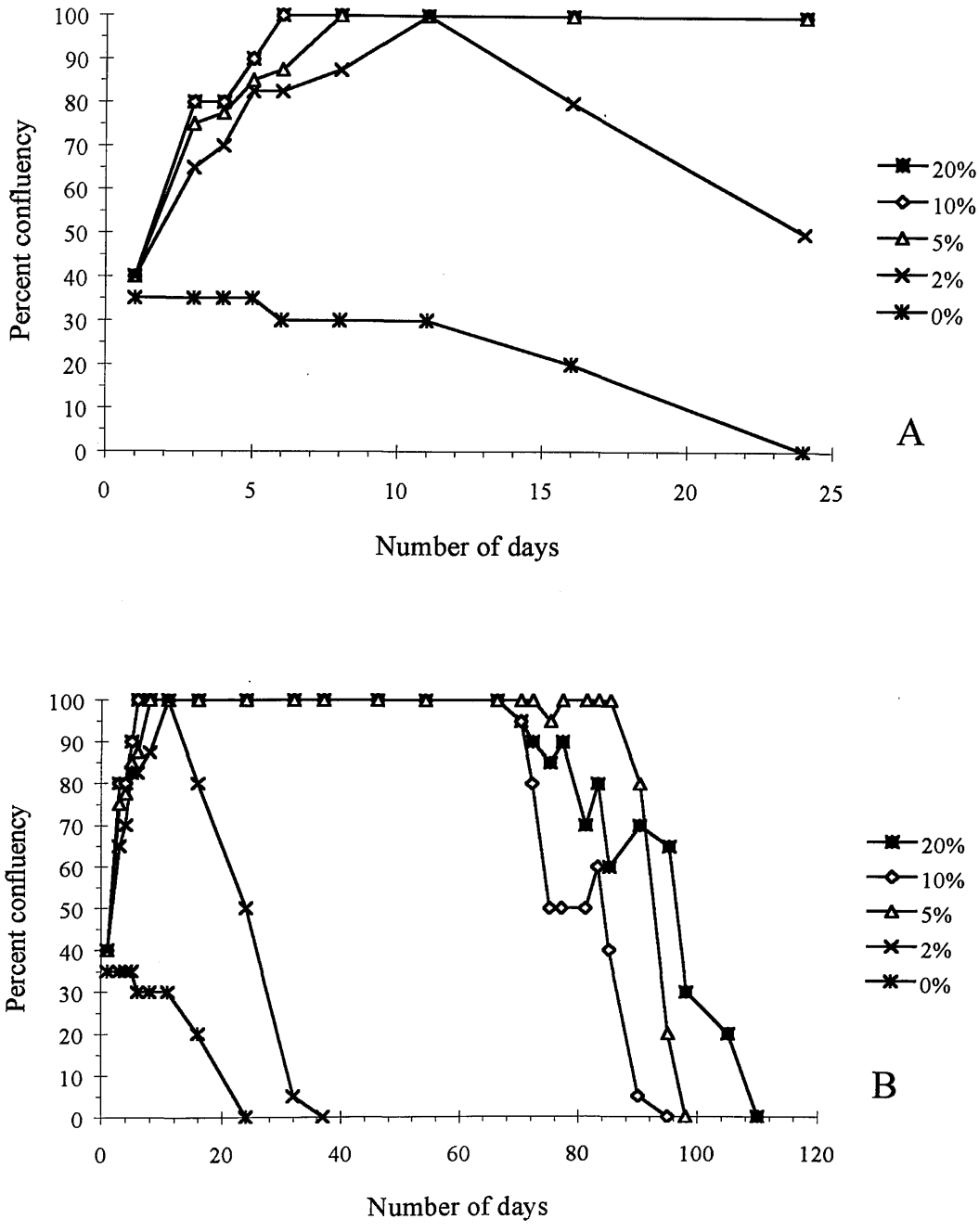


Figure 2.6 Growth of HCK passage 64 cultured in EMEM supplemented with 20%, 10%, 5% and 2% FCS and incubated at 28°C. HCK seeded at 3.4×10^5 cells/flask. (A) Growth up to 24 days (Note: Cells growth at 20% and 10% serum supplemented were similar.). (B) Growth up to 110 days.

HCT cells at passage 22 were also able to grow to cover the entire surface of the flask on days 8, 8, 5 and 5 in 2-20% serum supplemented media, respectively, after seeding at 4.0×10^5 cells/flask. The monolayers retained confluency for 31, 31, 39 and 50 days post-seeding in 2-20% serum containing media, respectively (Figure 2.7a-b). Both cell lines grew best in EMEM medium supplemented with 5-20% FCS but the HCK line retained confluent monolayers longer than the HCT line.

2.4.4 Cryopreservation

The initial viability of HCK passage 55 and HCT passage 18 cells prepared for cryopreservation in liquid nitrogen and for deep freezing at -70°C was 95.9% and 100%, respectively. After 24 h storage, the viabilities of the same cells taken from liquid nitrogen were 91.8% and 83.0% while those thawed from -70°C were 94.5% and 87.2%, respectively. Cultured HCK and HCT cells recovered from both liquid nitrogen and -70°C storage reached confluency within 2-3 days at 28°C. Cells stored in liquid nitrogen for 30 or 33 months still had high viabilities of 91.8-94.2% for HCK and 81.5-88.4% for HCT, and were able to grow and reach full confluency within 3-4 days. More limited storage stability at -70°C was found for both cell lines. HCK cells remained viable for at least 6 months but HCT cells could only be stored for less than 6 months in a deep freezer. After seeding into flasks, the time to achieve confluency seemed longer with increasing storage time. HCT cells thawed at 3 months had 43.2% viability and took 6 days to grow over the whole surface whereas at 6 months there was 40.4% viability and the cells did not adhere to the flask surface. Both cell lines thawed at 18 or 21 months from -70°C were found to be dead (Table 2.2).

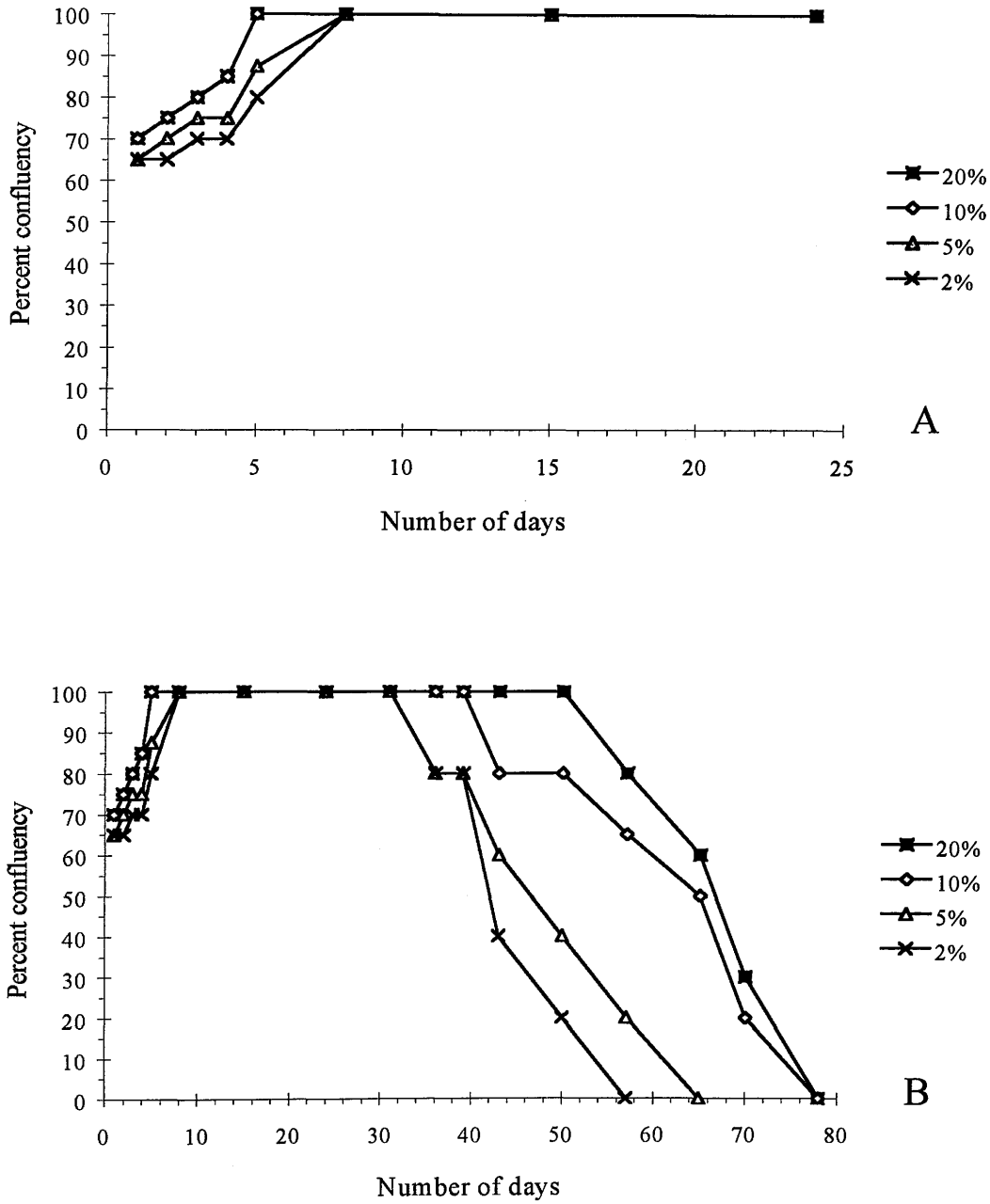


Figure 2.7 Growth of HCT passage 22 cultured in EMEM supplemented with 20%, 10%, 5% and 2% FCS and incubated at 28°C. HCT seeded at 4.0×10^5 cells/flask. (A) Growth up to 25 days (Note: Cells growth at 20% and 10% serum supplemented were similar.). (B) Growth up to 78 days.

Table 2.2 Viability and time to confluency of HCK passage 55 and HCT passage 18 cells thawed from liquid nitrogen and deep freeze at different storage times.

Cells & Storage time	Liquid nitrogen (-196°C)			Deep freeze (-70°C)		
	Total cell count (cells/ml)	Cell viability (%)	No. of days to achieve confluency	Total cell count (cells/ml)	Cell viability (%)	No. of days to achieve confluency
HCK						
1 day	1.22×10^6	91.8	2	1.28×10^6	94.5	2
1 month	nd	nd	nd	0.94×10^6	85.1	4
2 months	nd	nd	nd	1.05×10^6	85.0	5
4 months	nd	nd	nd	1.01×10^6	89.7	11
6 months	0.76×10^6	92.2	4	1.17×10^6	85.4	8
21 months	1.0×10^6	94.0	3	0	0	*
33 months	1.03×10^6	94.2	3	nd	nd	nd
HCT						
1 day	0.94×10^6	83.0	3	0.94×10^6	87.2	3
1 month	nd	nd	nd	0.84×10^6	85.5	4
3 months	0.90×10^6	81.5	3	0.88×10^6	43.2	6
6 months	nd	nd	nd	0.90×10^6	40.4	*
18 months	0.98×10^6	82.8	3	0	0	*
30 months	0.81×10^6	88.4	3	nd	nd	nd

* Cells did not achieve confluency and died.

nd = not done

2.4.5 Plating Efficiency

HCK passage 60 cells were seeded at 5.76×10^2, 5.76×10^3 and 5.76×10^4 cells/flask. At the two higher densities, cells grew and formed complete cell sheets. At the lowest density of 576 cells/flask, cells grew as single colonies by day 9 after seeding. Colony counts in 3 replicate flasks were 153, 201 and 205 with an average of 186 colonies. The efficiency of plating of HCK was calculated as 32.3%. For the HCT line passage 22, cells were seeded at 5.0×10^2, 5.0×10^3 and 5.0×10^4 cells/flask. Similar to HCK cells, only the lowest concentration of cells grew as single colonies which were fixed and stained on day 14 post-seeding. Colony counts in 3 replicated flasks were 42, 100 and 110 with an average of 84 colonies. The plating efficiency of HCT was 16.8%.

2.4.6 Microorganism Contamination

The HCK and HCT inoculated trypticase soy broth, Sabouraud dextrose broth and thioglycollate broth media showed no growth of any bacteria or fungi at 22°C and 37°C over 14 days observation, indicating both cell lines to be free from microbial contamination.

2.4.7 Mycoplasma Contamination

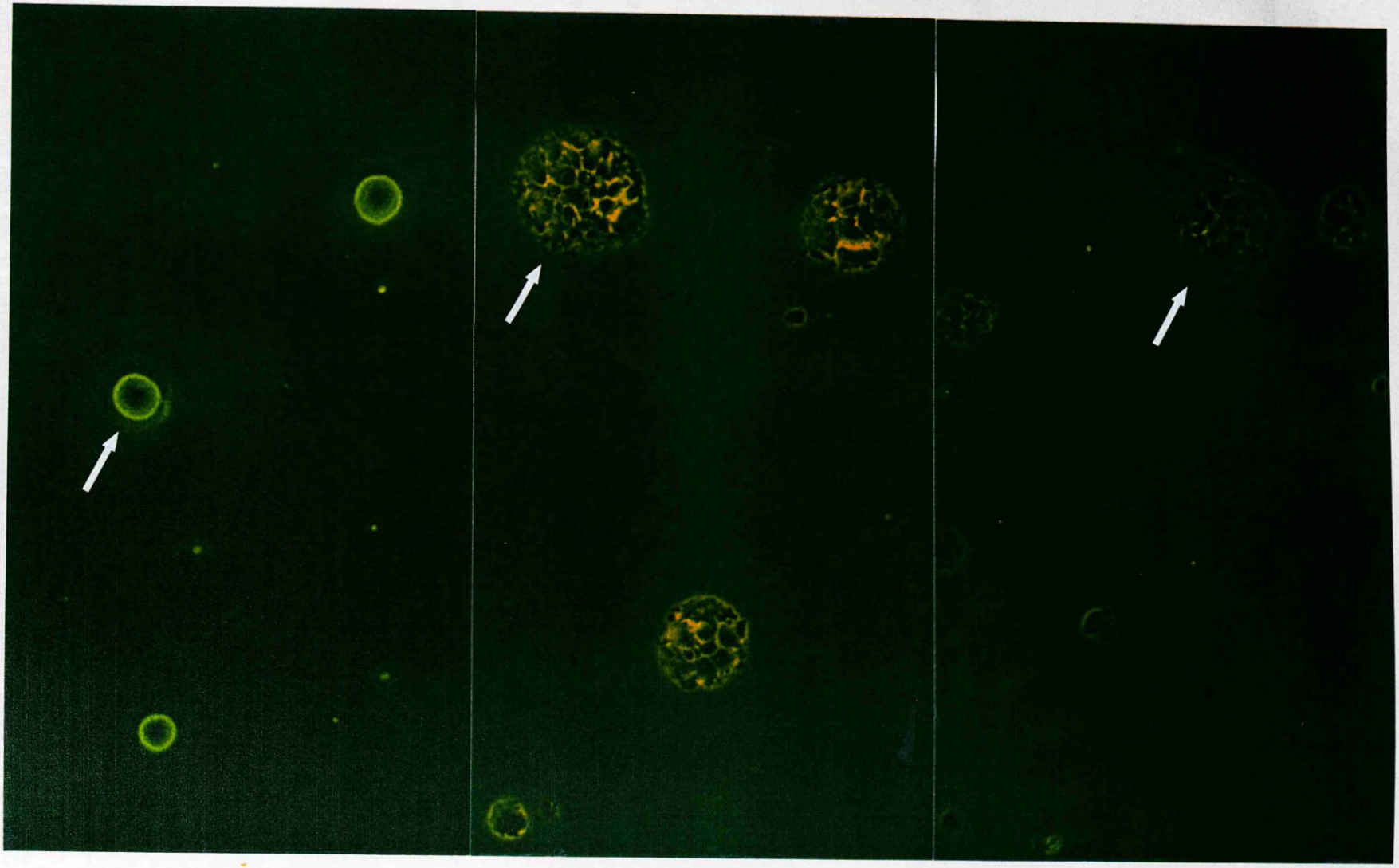
Mycoplasma isolation tests on HCK passage 60 and HCT passage 20 cells were found to be negative, while the positive control, CCSN passage 56, showed typical fried egg-like mycoplasma colonies on the mycoplasma agar plate (Figure 2.8).

Figure 2.8 Isolation of mycoplasmas after incubation at 37°C for 20 days. Magnification = ×90

a. Mycoplasma-contaminated CCSN cell line showed fried egg-like colony of mycoplasmas (arrow) on agar plate. Colonies started to appear 7-8 days post-inoculation.

b. Colony of HCK cells (arrow) on agar plate showed no mycoplasmas.

c. Colony of HCT cells (arrow) on agar plate showed no mycoplasmas.



The inoculation of cells into broth medium and subculture to the agar plate was found to isolate many more mycoplasma colonies than direct inoculation of cells on to the agar plate. Hoechst stain tests on both cell lines were negative when compared with the control slides (Figure 2.9a-d). These tests indicate no mycoplasma contamination in either cell line.

2.4.8 Chromosome Analysis

HCK passage 19 and passage 63 had similar modal chromosome numbers of 63-64 and 64-66, respectively. HCT passage 20 had a modal number of 55 (Figure 2.10a-c). At least one homologous chromosome of HCK cells appeared in triplicate indicating aneuploidy while the HCT line was diploid. Chromosome morphology for both cell lines is shown in Figure 2.11a-b.

2.4.9 Adventitious Viruses

No adventitious viruses were identified from HCK passage 65 and HCT passage 24 cells. HCK and HCT cells did not induce any specific cytopathic changes in EPC, FHM, BB or SSN-1 cell cultures.

2.4.10 Susceptibility to Virus Infection

Sixteen viruses were used to test the susceptibility of HCK and HCT cell lines (Table 2.3). HCT was refractory to all 7 EUS-associated rhabdoviruses used. HCK was also

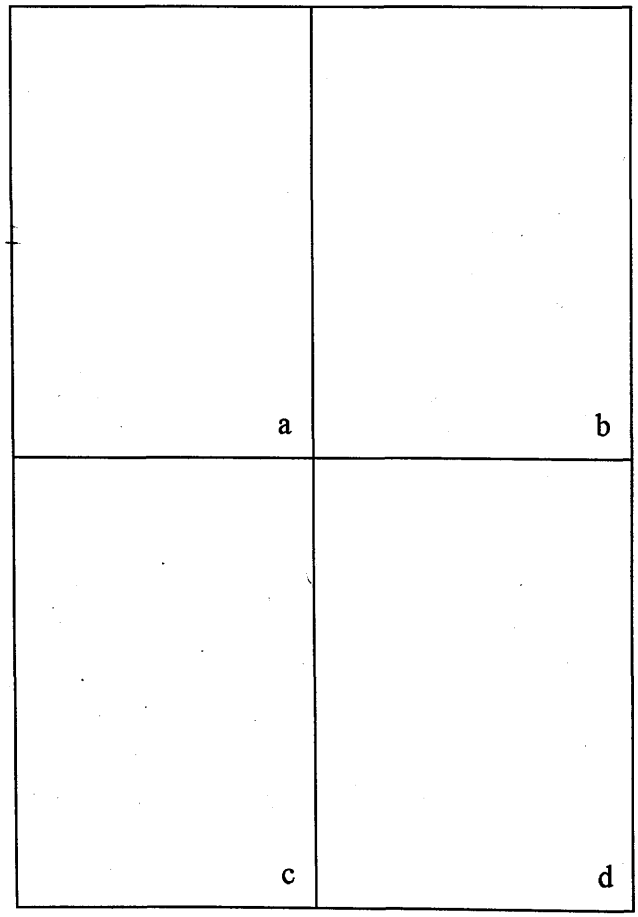
Figure 2.9 Mycoplasma identification in cell culture using Hoechst stain. Magnification = ×370

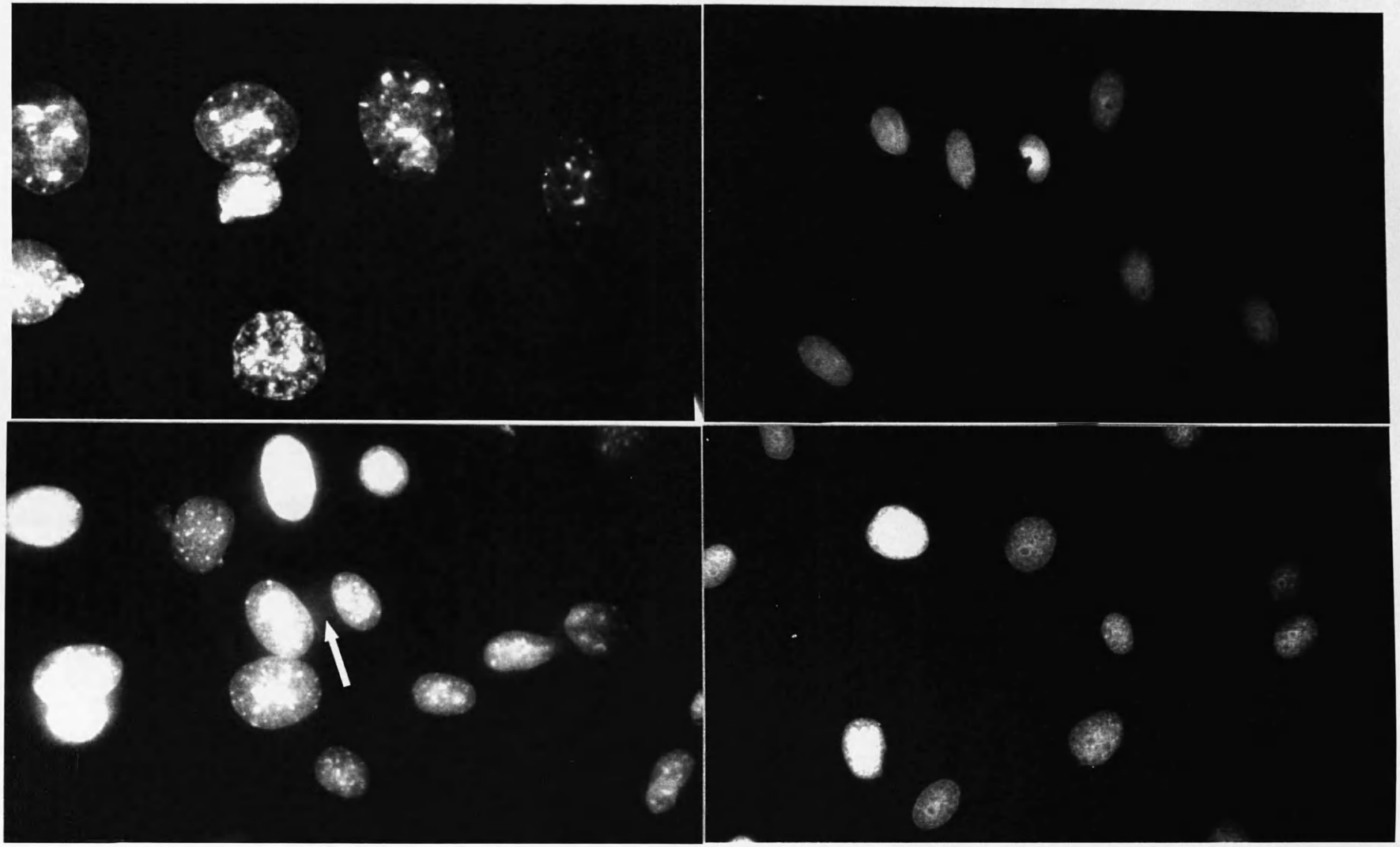
a. Positive control, mycoplasma-infected 3T-6 mouse cells showing tiny dots of nuclear DNA of mycoplasma in host cytoplasm (arrow).

b. Negative control, 3T-6 mouse cells showing no mycoplasma.

c. HCK passage 65 showing no mycoplasmas.

d. HCT passage 20 showing no mycoplasmas.





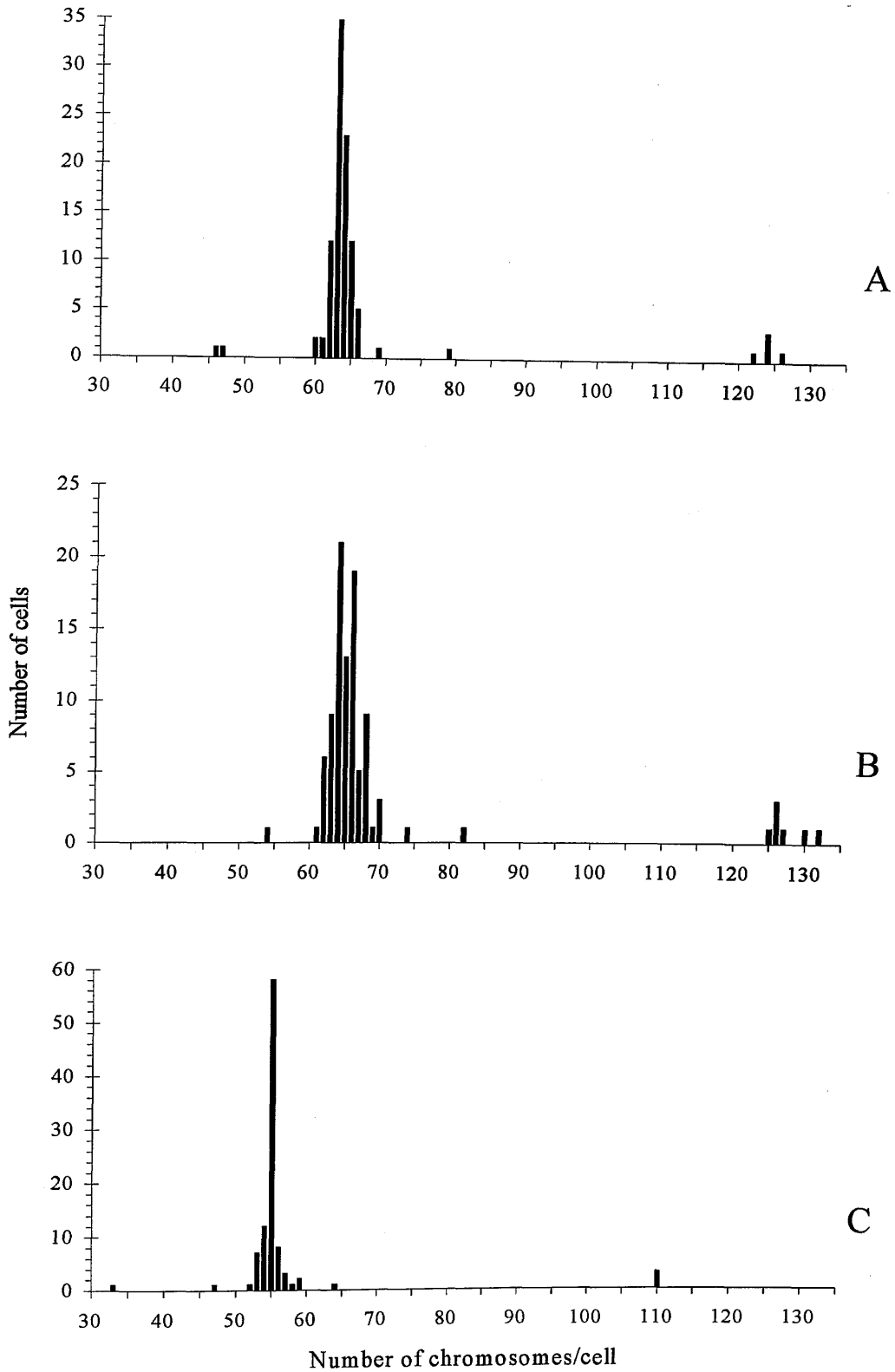


Figure 2.10 Distribution of chromosomes in HCK and HCT cells. (A) HCK at passage 19 had aneuploid chromosomes 2n = 63-64. (B) HCK at passage 63 had aneuploid chromosomes 2n = 64-66. (C) HCT at passage 20 had diploid chromosomes 2n = 55.

Figure 2.11 Chromosome spread obtained from hybrid catfish cell lines. The metaphase was blocked using 0.5 μg/ml colcemid for 4 h. Cells were fixed, swollen using hypotonic solution, dropped on to slides and stained with Giemsa. Bar = 5.3 μm

a. HCK passage 63 presented aneuploid chromosomes 2n = 65. The largest acrocentric chromosomes had 3 homologous chromosomes (arrows) that indicated aneuploidy.

b. HCT passage 20 with diploid chromosomes 2n = 55. a = artifact

a
a
a

Table 2.3 Susceptibility of HCK and HCT lines to 16 fish viruses. The amount of inoculated virus was determined by titration on permissive cell lines. Tests conducted in 24-well plates at 20°C. First passage performed on day 9 or 10 after initial inoculation.

Viruses	Cell lines*	Log_{10} $TCID_{50}$/ 1 ml virus	Susceptibility of HCK			Susceptibility of HCT		
			initial	1st pass	2nd pass	initial	1st pass	2nd pass
Rhabdoviruses								
T9204	SSN-1	8.20	+	+	nd	–	–	nd
20E	SSN-1	6.02	–	–	nd	–	–	nd
O2	SSN-1	5.44	–	–	nd	–	–	nd
SL11	SSN-1	5.22	–	–	nd	–	–	nd
BP	SSN-1	7.19	–	–	nd	–	–	nd
19	SSN-1	5.09	–	–	nd	–	–	nd
A4	SSN-1	6.14	–	–	nd	–	–	nd
Birnaviruses								
IPNV Sp	CHSE-214	8.80	+	+	+	+	+	nd
IPNV Ab	CHSE-214	8.20	+	+	+	+	+	nd
SGV	CHSE-214	7.12	+	+	+	+	+	nd
Herpesvirus								
CCV	BB	7.47	?	?	–	?	–	–
Reoviruses								
GSV	FHM	7.48	?	+	+	?	+	+
CRV	BB	6.14	?	+	+	?	?	+
Tench	FHM	8.12	+	+	+	?	–	–
Chub	FHM	7.40	+	+	+	?	–	–
reovirus-like T9231	SSN-1	8.77	–	–	nd	–	–	nd

* SSN-1 line derived from whole fry of striped snakehead (*Channa striata*) (Lilley and Frerichs, 1994)
CHSE-214 line derived from chinook salmon embryo (Nims *et al.*, 1970).
BB line derived from brown bullhead caudal trunk (Wolf and Quimby, 1969).
FHM line derived from fathead minnow tail (Gravell and Malsberger, 1965).
initial = initial inoculation; + = sensitive; – = resistant; ? = doubtful;
nd = not done.

resistant to infection with 6 strains isolated between 1985-1988 (strains 20E, O2, SL11, BP, 19 and A4) but was sensitive to the 1992 isolate (strain T9204). Rhabdovirus T9204 caused rounding up and clustering of affected cells within 4 days post-inoculation. The three birnaviruses caused similar changes on both lines but the times to complete CPE of infected HCK cells was faster than for HCT cells. HCK cells started to develop a CPE on days 1-2 and showed complete CPE on days 2-4 post-infection. Clustering and rounding up of affected cells were seen and followed by cell lysis which left a large residue of cell debris. In HCT, many rounding up cells and a few swollen cells were first observed on day 2 or 3. These changes gradually progressed and complete CPE was observed on days 7-10 post-infection (Figure 2.12a-d). IPNV serotype Sp caused more rapid changes than the other two viruses in the same family.

Differences were found between the susceptibility of the 2 lines to 4 reoviruses. The HCK cell line was sensitive to golden shiner reovirus (GSV), channel catfish reovirus (CRV) and tench and chub reoviruses whereas the HCT cell line was only susceptible to GSV and CRV. GSV and CRV produced very little change in HCK and HCT cells following initial inoculation. However, both these reoviruses started to cause rounding up and clustering of affected cells on first or second passage at day 3 for HCK and day 8 for HCT. Complete CPE for CRV-infected HCK and HCT did not develop during 20 days observation (Figure 2.12e). Tench and chub reoviruses caused a distinct CPE between 2-5 days post-inoculation beginning with cell elongation to rounding up and finally lysis following a small residue of cell debris (Figure 2.12f). Both lines were refractory to infection with herpesvirus (channel catfish virus : CCV) and a recently

Figure 2.12 Cytopathic effects in HCK and HCT lines caused by virus infection. Bar = 120 μm

a. Control HCK passage 75.

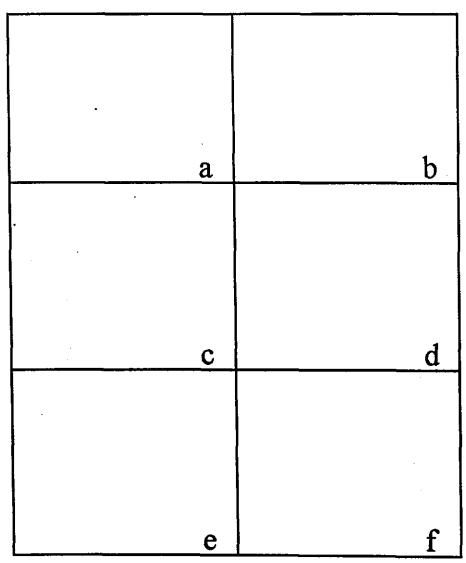
b. Control HCT passage 31.

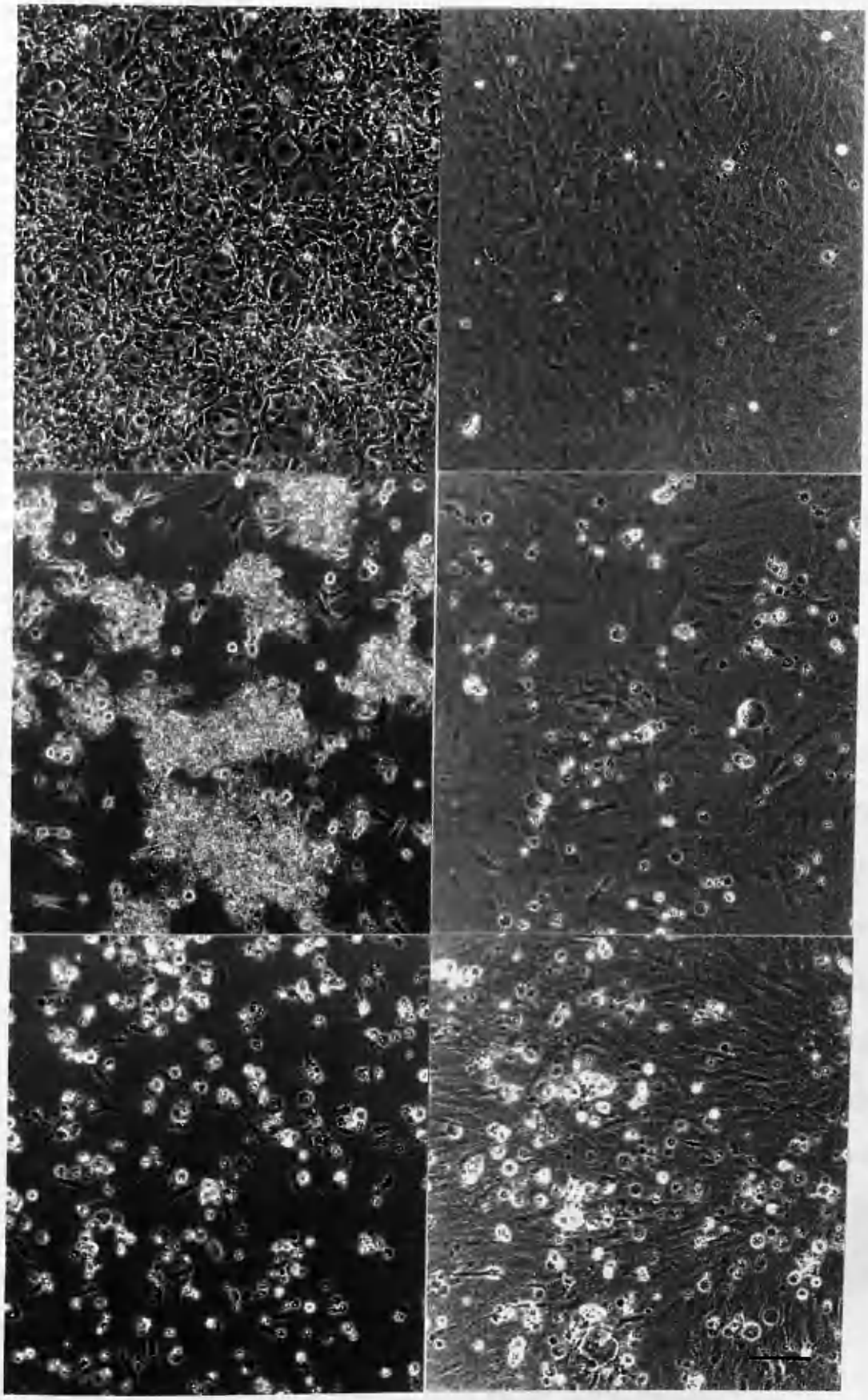
c. HCK passage 75 cells infected with IPNV serotype Sp on day 3 showing complete CPE with rounding-up of cells and a large cluster of affected cells.

d. HCT passage 31 cells infected with IPNV serotype Sp on day 3 post-inoculation showing early CPE with many rounding-up cells and a few swollen cells.

e. HCK passage 22 cells infected with tench reovirus on day 4 post-inoculation showing complete CPE with rounding-up cells. The HCK control cells were normal.

f. HCK passage 22 cells infected with CRV on day 9 post-infection showing CPE with many rounding-up cells. Control cells were normal.





isolated EUS-associated reovirus (T9231). Although CCV induced some changes following the initial inoculation, the changes did not continue on passage.

2.5 Discussion

Two new cell lines designated HCK and HCT derived from hybrid catfish have now been established following initiation of primary cultures in 1991 and 1992, respectively. The presence of desmosomes, in monolayer cultures derived from tail tissue, indicated HCT to be an epithelial cell type (Garrod, 1986; Bazzola and Russell, 1992). Many other lines originating from fin, peduncle or tail of fishes have also been found to be of epithelial-like cell type (Wolf and Mann, 1980). HCK derived from head kidney was a fibroblast-like cell line with cell morphology similar to a line derived from the kidney of walking catfish, *Clarias batrachus*, (Noga and Hartmann, 1981). The present study found electronmicrography to be very useful for cell type identification but such facilities are very expensive. Without electronmicroscopy, Cowin and Garrad (1983) demonstrated desmosomes in tissue using an immunofluorescent technique with polyclonal antibodies against desmosomal protein and glycoprotein in which the protein antigens had been purified by gel electrophoresis. It was found that epithelial cells of reptile (lizard), amphibian (frog), fish (trout) and mammal (human, cow, guinea pig, rat) shared similar desmosomal antigens (Cowin *et al.*, 1984). It would be interesting to evaluate the similarity of desmosomal proteins in epithelial cells of different fish cell lines using the immunofluorescent technique. If common protein antigens were present, a standard test could be developed for fish cell culturists to identify cell line types.

To date, HCK and HCT have been subcultured for 83 and 42 passages, respectively. HCK has a greater tolerance to a wide range of temperatures with cells able to grow from 22-37°C and retaining monolayer confluence for at least 14 days at 37°C. A high temperature tolerance has been found for most warm-water fish cell lines (Nicholson, 1989). HCT grew best at 22-28°C but degenerated rapidly when incubated at 37°C. Both lines grew best in media supplemented with 5-20% serum but the HCT epithelial cell type had the ability to grow in EMEM with 2% serum better than the HCK fibroblast cell type. However, slow growth of cells in serum-reduced medium may be increased by adding proteins and hormones to the culture medium as reported for cell cultures of lake trout, *Salvelinus namaycush* (Cheng *et al.*, 1993). These authors also commented that a high serum (15-20%) concentration in the culture medium may have caused the failure of a previous attempt to develop lake trout cell cultures. The effects of serum in media for higher vertebrate cell cultures have been documented by Lechner (1985). It was reported that serum could accelerate growth of fibroblast cells in primary cultures such as mesenchymal fibroblast cells but inhibited or altered some characteristics of lines such as rat thyroid follicle cells. An awareness of toxins, viruses and mycoplasmas being carried in serum and a need for special media to study specific biochemical mechanisms of eukaryotic cells has increased the importance of serum-free media (Taylor and Parshad, 1977; Bjare, 1992). Shea and Berry (1983) reported the use of a serum-free medium in a fish cell culture system. Five piscine lines, CAR, FHM, EFC, CHSE-214 and ABIII (un-established line of goldfish swimbladder) were found to grow in serum substitute containing Medium 199 as well as 10% serum containing Medium 199.

HCK and HCT lines could be stored in liquid nitrogen for longer than 30 months with high cell viability post-thawing but deep freeze storage could maintain both lines for only 3-6 months. The latter times were short compared to over 12 months storage time for a Japanese flounder cell line (Meguro *et al.*, 1991) and 9 salmonid cell lines (Lannan *et al.*, 1984). A channel catfish ovary (CCO) line also had a short storage time in the deep freezer. When CCO cells were thawed after 1 month, cell viability was found to be only 36% (Bowser and Plumb, 1980). However, an Atlantic salmon cell line (AS) was reportedly of inconsistent viability after thawing from the deep frozen state (Nicholson and Byrne, 1973). Andral *et al.* (1990) also found some difficulty in culturing a thawed rainbow trout kidney cell line. Surprisingly, rainbow trout gonad (RTG-2) cells were still viable and could be recovered after 10 years storage at -80°C (Wolf and Mann, 1980). HCK and HCT had 32.3% and 16.8% plating efficiency and split ratios 1:3 and 1:2 were suitable for routine subculture. The plating efficiencies of fish cell lines seem to have a wide range with values from 7% to 28% found for 14 warm-water cell lines (Chen and Kou, 1988). Lu *et al.* (1990) reported that 3 cell lines of Chinese carp, *Ctenopharyngodon idella*, had plating efficiencies ranging from 4-14%. A similar wide range, 7-29%, was also reported for 3 cell lines of marine fishes (Fernandez *et al.*, 1993a). A very high value of 48% was found for a cell line derived from an epithelioma tumor of gold fish (Fernandez *et al.*, 1993b).

Throughout the period of this study, both cell lines remained free from micro-organisms and mycoplasma contamination. No adventitious viruses were observed in 4 indicator cell lines and no virus-like particles were seen in electronmicrographs. Based on a karyology report for newly hatched larvae by Lawonyawut *et al.* (1992) the same hybrid

catfish has diploid chromosomes 2n = 55. HCK passages 19 and 63 showed similar aneuploid chromosomes 2n = 63-64 and 2n = 64-66 as some chromosome pairs seemed to have one extra copy (Faisal *et al.*, 1992) while HCT passage 20 had diploid chromosomes 2n=55. The chromosome number of HCK differed from that of HCT for reasons which are not presently known. Hybrid catfish donors obtained from different breeding crops may account for the difference. However, if the suggestion that karyology in fish cell lines should be performed at a passage level less than 4 in order to obtain an accurate chromosome identification of the species (Amemiya *et al.*, 1984) is also true for hybrid catfish, the HCK cell line may have experienced *in vitro* genomic transformation before passage 19. Karyotyping of both cell lines was found difficult because of the similarity of the Giemsa-stained chromosomes. Further work needs to be done using chromosome banding techniques in order to distinguish each set of homologous chromosomes and confirm aneuploidy of the HCK line (Almeida-Toledo *et al.*, 1988; Delany and Bloom, 1984)

HCK would appear to have greater potential value for future virological studies than HCT as HCK proved to be susceptible to a slightly wider spectrum of viruses which comprised 3 birnaviruses, 4 reoviruses and 1 EUS-associated rhabdovirus. Surprisingly, among the 7 EUS-associated rhabdoviruses, which were of principal interest, only the HCK line was sensitive to a single isolate, T9204. This result indicates that isolate T9204 differs from the other rhabdoviruses in support of the finding of Lilley and Frerichs (1994). Both lines were sensitive to SGV birnavirus but not to T9231 reovirus which had both been isolated from EUS diseased fishes in Thailand (Hedrick *et al.*, 1986; Roberts et al., 1994). All three birnaviruses (SGV and 2 serotypes of IPNV)

caused rapid cytopathic changes in both hybrid catfish cell lines when compared to the other viruses tested. The rapid CPE may indicate rapid propagation of virus particles. IPN viruses originally isolated from cold-water fishes were able to infect 13/14 warm-water fish cell lines (Chen and Kou, 1988) as well as the 2 cell lines in this present study. CCV induced some changes in HCK and HCT lines at 20°C. It would be interesting to test CCV susceptibility at 25-30°C as 30°C induced maximum replication activity of CCV (Bowser and Plumb, 1980). Furthermore, 3 cell lines derived from walking catfish, *Clarias batrachus*, the same genus as the hybrid catfish parent, were susceptible to CCV at 25°C (Noga and Hartmann, 1981).

Only the HCK line was sensitive to tench and chub reoviruses but no plaque-like CPE was induced. However, the stock reoviruses prepared in the FHM line showed many focal syncytia with rounded cells at the edge of the plaques similar to that reported by Ahne and Hölbl (1988). The stock preparation of GSV also caused focal degeneration in the FHM line similar to tench and chub reovirus and similar to the report of Schwedler and Plumb (1982). However, GSV induced a different CPE in HCK cells. GSV-infected HCK cells showed rounded-up and swollen cells and clusters of affected cells. GSV-infected HCT cells showed similar changes to HCK but cell lysis and clusters of cell debris were dominant. CRV induced transient CPE in both cell lines with rounding-up and small clusters of cells but not complete CPE. However, complete CPE was found in CRV-infected BB line in which the stock virus was grown. It has been noted that different passages of reovirus may not cause the same CPE in an infected cell line (Winton *et al.*, 1987).

It is intended that the HCK cell line will be used in conjunction with other cell lines known to be susceptible to EUS associated viruses in further virological studies of EUS in Thailand. Apart from virological studies, the 2 hybrid catfish cell lines can be used in many fields of research and as a teaching tool in many subjects in high school or university (Nicholson, 1989).

Chapter 3

Field Observations on Epizootic Ulcerative Syndrome-diseased Fishes and Virus Isolation

3.1 Introduction and Literature Review

EUS is a seasonal disease condition affecting wild and cultured fishes in most Asian countries. A virus was soon recognised as a possible causative agent as the disease spread widely after heavy rain and in the cool period of the year, and viruses had been visualized and/or isolated from EUS-diseased fishes (Roberts *et al.*, 1986). An FAO expert consultation on EUS was concerned that disease diagnostic capabilities especially in the field of virology were limited because of a lack of virology laboratories and personnel dealing with fish virology in southeast Asian countries and recommended that a central fish disease institution with fully equipped facilities needed to be located in the region (FAO, 1986). In 1991, the suggested fish disease institute was established in Bangkok as the Aquatic Animal Health Research Institute (AAHRI) with fully equipped facilities for studies in many fields of fish diseases including virology. The establishment of AAHRI was funded partly by ODA, United Kingdom, and partly by the Royal Thai Government. The new fish virology laboratory became operational for the first time in 1993 for virus isolation from the EUS-diseased fishes of this present study. The following earlier virological findings from EUS outbreaks provided the main points of interest for this study.

Scientists from the Faculty of Veterinary Science of Chulalongkorn University, Bangkok, were pioneers in the virological studies of EUS in Thailand. In 1982-1983, intracytoplasmic inclusions were observed in hepatocytes and renal tubule cells of affected fish (Rattanaphani *et al.*, 1983). Virus-like particles with a spherical shape measuring 70 nm were found in the liver of snakehead fish collected from Suphanburi province, central Thailand (Wattanavijarn *et al.*, 1983a). Wattanavijarn *et al.* (1983b) also found virus-like particles with pleomorphic forms which were coated with an envelope-like structure and located in muscle, spleen and endothelial cells of blood capillaries of snakehead fish collected from Suphanburi and Patumtani provinces. These workers suggested that particles could spread to any organ via blood vessels and could spread to other disease areas via birds, fish migration or the water supply. Similar virus-like particles were again observed in the liver and spleen of walking catfish, *Clarias batrachus*, collected from Nakorn-Pathom and Samut-Prakan provinces, central Thailand (Wattanavijarn *et al.*, 1984b).

The first virus isolation was attempted by Tangtrongpiros *et al.* (1983b) using the same samples in which Wattanvijarn *et al.* (1983b) had previously found virus-like particles under transmission electron microscopy. The samples had been frozen at -20°C for 5 months. A pooled tissue extract of liver, spleen and head kidney was inoculated on to EPC cell line cultures and an unestablished snakehead fish cell line derived from head kidney and incubated at 22°C. Following initial inoculation, a cytopathic effect (CPE) was observed from days 2-5 (start-complete) for the EPC line and days 2-7 for the snakehead fish line. A transmissible CPE was obtained on the next 2 passages. Two years later, Wattanavijarn *et al.* (1986) reported the successful isolation of 2 different

viruses, a rhabdovirus and a birnavirus, from diseased snakehead fish, catfish (*Clarias* sp.), sand goby (*Oxyeleotris marmoratus*), swamp eel (*Fluta alba*), snakeskin gourami (*Trichogaster pectoralis*), hard-lipped barb (*Osteochilus hasselli*), catfish (*Mystus nemurus*) and silver barb (*Puntius gonionotus*). Four cell lines, RTG-2, BF-2, FHM and the unestablished snakehead fish line, were inoculated with tissue extracts and a complete CPE obtained within 5-7 days. The rhabdovirus was identified by Ahne *et al.* (1988) and shown to be serologically different to 5 known fish rhabdoviruses. The same rhabdovirus was later characterised and named as snakehead rhabdovirus (SHRV) by Kasornchandra *et al.* (1991). The birnavirus isolate was later identified by Wattanavijarn *et al.* (1988) as serologically similar to IPNV serotype Sp.

Saitanu *et al.* (1986) carried out virus isolation studies in EUS-affected snakehead fish, giant snakehead fish (*Channa micropeltes*), sand goby, three-spot gourami (*Trichogaster trichopterus*), striped croaking gourami (*Trichopsis vitatus*), Siamese fighting fish (*Betta splendens*) and wrestling half-beak (*Dermogenus postillus*). The snakehead fish were collected during the 1982-83 epizootic and the other species during the 1983-1984 outbreak. Most of the tissue extract samples caused CPE within 3-7 days after inoculation in BF-2, BB and FHM lines at 25°C. The virus isolate was named snakehead virus (SHV) and was similar in size and shape to IPNV birnavirus.

During the 1983-84 EUS outbreak another birnavirus was isolated from diseased sand goby using BF-2, EPC and FHM cell lines (Hedrick *et al.*, 1986). The sample was collected from cage culture in Chaophrya River, Ayudhaya province. This birnavirus

was named SGV and showed characteristics different from other known fish viruses of the Birnaviridae family.

Frerichs *et al.* (1986) reported successful virus isolations from diseased fishes collected from Chiang Mai and Udornthani provinces of Thailand and Rangoon city of Myanmar during the 1985-86 epizootic. All isolates were identified as rhabdoviruses by morphology in electromicrographs. The finding of a rhabdovirus in different areas suggested an important role for the virus in the disease outbreak (Frerichs *et al.*, 1986). Details of these findings and new rhabdovirus isolates were given by Frerichs *et al.* (1989b). They also reported the results of attempted virus isolation from 94 diseased and 46 normal fishes collected during 1985-1988 from Thailand, Myanmar, Malaysia, Indonesia, Lao P.D.R., the Philippines and Sri Lanka. Most of the selected tissue samples were placed in transport medium [Hank's balanced salt solution (HBSS) supplemented with 10% FCS and antibiotics] and sent to the Institute of Aquaculture where virus isolation was performed. Only 6 rhabdovirus isolates were obtained from diseased fishes, 3 from Thailand, 2 from Myanmar and 1 from Sri Lanka. The name ulcerative disease rhabdovirus (UDRV) was adopted for these viruses which have been further studied and characterised by Frerichs *et al.* (1989a), Kasornchandra *et al.* (1992b) and Lilley and Frerichs (1994).

During the early period of an EUS outbreak in 1991-1992, another rhabdovirus and a reovirus-like agent were recovered from EUS-affected snakehead fish from Suphanburi province, Thailand (Roberts *et al.*, 1994). This latter rhabdovirus was included in the

characterisation studies of Lilley and Frerichs (1994). Another birnavirus was isolated from diseased giant snakehead fish in Singapore (Subramaniam *et al.*, 1993).

Kasornchandra *et al.* (1988) developed a snakehead fin cell line (SHF) which was used for virus isolation studies from EUS-diseased fishes collected during the late period of EUS outbreaks in April 1989 in Assam, Tripura and West Bengal, India. No viruses were recovered (Boonyaratpalin, 1989b).

Prior to the present study, 4 birnaviruses, 8 rhabdoviruses and 1 reovirus have been isolated. Although a virus is believed to be a primary causative agent, the percent successful virus isolation was very low (Roberts *et al.*, 1994). For samples sent to the UK in particular, it is possible that any virus in tissue samples may have been lost during transportation from Asia to the UK laboratory as it might take days before samples are processed and inoculated. It is very important to keep samples cooled during transportation and they should be processed and inoculated on to susceptible cell lines within 2 or 3 days in order to obtain the best results (Amos, 1985). The percentage virus isolation from the laboratory in Chulalongkorn University, Bangkok, was not given. However, Saitanu *et al.* (1986) stated that they could isolate viruses from most of the tissue samples.

It is unusual to isolate 3 virus families from the same disease and difficult to say all or which of them may be pathogenic agents. Nevertheless, Jensen *et al.* (1979) reported the isolation of 2 different viruses, a rhabdovirus and an iridovirus, from Atlantic cod with ulcus syndrome in Western Europe. Jørgensen (1982a) also isolated 2 viruses, a

rhabdovirus and a birnavirus from natural stream water rainbow trout in Denmark. It is therefore important that further virus isolation studies from EUS-affected fishes are carried out in order to determine the significance of viruses in EUS outbreaks. There is a need to verify which virus is most frequently isolated among those 3 virus families. The cell line derived from snakehead fish fry, SSN-1, was found suitable for virus isolation from EUS-affected fishes (Frerichs *et al.*, 1989b). It has also been shown (Chapter 2) that the HCK line is sensitive to rhabdovirus infection. BF-2 and SSN-1 lines were susceptible to 5 EUS rhabdovirus strains (Frerichs *et al.*, 1989b) and EPC was susceptible to EUS virus strain SHRV (Kasornchandra *et al.*, 1991). These 4 lines were therefore selected for use in the present virus isolation study.

3.2 Objectives:

- To attempt virus isolation from EUS-affected fishes at different periods of the 1993-1994 epizootic
- To compare the ability of HCK, SSN-1, BF-2 and EPC cell lines to isolate EUS-associated viruses

3.3 Materials and Methods

EUS Outbreaks in Thailand, 1993-1994

During 1993-1994, EUS was first observed on 30th December 1993 in Suphanburi and on 1st January 1994 in Bangkok provinces. The disease occurred 5-7 days after the coldest period of the year when the water temperature was believed to be less than

25°C. The temperature then gradually increased and the cold period had ended by 2nd – 3rd weeks of January.

Fish with classic EUS lesions were collected from culture ponds in Bangkok, Suphanburi and Prachinburi provinces. The first 2 areas are located in central Thailand and the third in the East. In Suphanburi and Prachinburi, disease outbreaks first noted in the rice field irrigation canals and then spread to the fish culture ponds. In Bangkok, EUS outbreaks occurred initially in rice paddy field ponds.

Tissue Preparation

The semi-sterile technique was used except where stated. Tissue preparation was similar to the general procedures stated elsewhere (Amos 1985; Hetrick, 1989). Brief details are as follows:

Fish were sacrificed and wiped clean with tissue paper. Approximately 1 g of each tissue was sampled from muscle and internal organs. For muscle samples, tissue debris and surface fungus on the ulcerated lesions were removed using a clean razor blade. Pieces of muscle tissue were taken from beneath the lesions. For internal organ samples, the abdomen was carefully opened using clean scissors, and small pieces of tissue from kidney, liver, spleen, intestine and pancreas were taken and pooled.

Samples were homogenised using a sterile, pre-cooled pestle and mortar until a smooth paste was obtained. Sterile fine sand was added to facilitate homogenisation. Samples were diluted 1:10 by the addition of 9 ml HBSS containing 2% FCS. After mixing well,

the samples were transferred to sterile centrifuge tubes and spun at 2500 rpm (800×g) at 4°C for 15 min to separate cell debris, sand and possibly some contaminating micro-organisms from the fluid extract. A further 1:5 dilution was carried out by filling 5 ml sterile disposable syringes with 4 ml HBSS (2% FCS) and then drawing up 1 ml supernatant. These 1:50 final dilutions were mixed well then filter sterilised through 0.45 µm disposable filter units. The filtrates were kept in 5 ml sterile bottles at 4°C and inoculated onto selected cell lines within 3 days.

Virus Isolation

Simultaneous cell culture and sample inoculation was carried out using HCK, BF-2, SSN-1 and EPC cell lines. At least 2 different lines were used at the same time for each sample. Tests were conducted in 24-well plates. Each plate was first seeded with a single cell suspension of the indicator cell line in L-15 medium containing 2% FCS and 1× antibiotics. Each well received 1.3-1.4 ml of cell suspension. Cell density was sufficient to produce a 80-90% confluent monolayer 1 day after seeding. Tissue samples (1:50 dilution) were immediately inoculated into 2, 3 or 4 replicate wells as appropriate. The inoculum volume was 200 µl/well. The same number of replicate wells remained as negative controls for each plate. Cells were incubated at 23°C and observed daily for CPE for at least 14 days. A first blind passage of culture fluids was performed on day 10 by transferring 200 µl of supernatant from each well to fresh culture wells and observing the plates for a further 14 days. A second blind passage was also carried out and a third blind passage was performed for some samples.

Samples showing cytopathic effects in which the cell monolayer changed, disintegrated, sloughed off the surface of the tissue culture wells and ended with cell lysis, were passaged to provide larger quantities of suspect virus. One ml of supernatant from a single well exhibiting CPE was inoculated into 25 cm^2 flasks containing a 80-90% confluent cell monolayer. The suspected virus was allowed to adsorb for 1 h. The cells were washed once with 5 ml PBS then 7 ml of maintenance medium was added. Flasks were incubated at 23°C together with un-inoculated control flasks for comparison. When the cells showed complete CPE, they were spun at 2500 rpm (800×g) at 4°C for 15 min. The supernatants were collected, aliquoted in 1 ml quantities and stored at -70°C for further characterisation. Successful virus isolation was confirmed by the continued development of a CPE over 2 or 3 passages.

3.3.1 Early Period EUS

Early period EUS fish samples were collected on 4th, 7th and 11th January 1994, within 2 weeks of the start of the outbreak. Samples were collected from Bangkok and Suphanburi Provinces as follows:

- **Bangkok :** The disease site was a rice paddy field polyculture (many fish species) pond with an extensive culture system (low stocking density and no feeding). The rice paddy field covered an area of 18.5 rai (1 rai = 1600 m^2). Water in the field was drained in the middle of December 1993 to harvest the rice and fishes gathered in 1 pond size 6 × 100 × 2.5 m^3 (width × length × depth) located on 1 side of the rice field. Many fish species were present including striped snakehead, three-

spot gourami (*Trichogaster trichopterus*), common carp (*Cyprinus carpio*), nile tilapia (*Oreochromis nilotica*) and barb (*Puntius* spp.). Only the first 2 species were affected by EUS. Over 30% of the total fish population in the pond had been lost by the time of sampling (Khun Looan, farmer, pers. comm.) . Six striped snakehead and 3 three-spot gourami fishes were collected on 7th January 1994.

- **Suphanburi site 1 :** This area incorporated 40 fish ponds (size ~1.5 rai/pond). Twenty-two ponds cultured snakehead fish, 15 cultured hybrid catfish and 3 ponds cultured silver barb (*Puntius gonionotus*). These were monoculture (single fish species) ponds with an intensive culture system (high stocking density and large amount of feeding) and shared the same water supply (continuous flow by gravity) from an irrigation canal. EUS-affected striped snakehead were first seen in this canal. The fish farm manager immediately stopped water running and applied lime (calcium hydroxide) at 60 kg/rai to the ponds in an attempt to prevent the spread of disease. Unfortunately, this did not appear to have been carried out early enough and EUS affected 1 striped snakehead pond. The disease was limited to this single pond but caused severe infection with 50% of the fish population affected by day 5, gradually increasing to 70% by day 12 (Khun Chokchai, farm manager, pers. comm.). Affected fish were 6 months old with an average weight of 250 g. Fish from this pond were taken for virus isolation studies on 4th and 11th January 1994.

3.3.2 Middle Period EUS

Middle period EUS fish samples were collected on 8th February 1994, 5 weeks after the outbreak started. They were obtained from Suphanburi Province as follows:

- **Suphanburi site 2 :** Site 2 is located 2 km to the north of site 1. Site 2 also had 40 ponds and used the same intensive culture and water system as site 1 but cultured only striped snakehead. The water supply to both sites came from the same irrigation canal. Water in the canal moved downstream passing through site 1 and then site 2. The farm managers of both sites first found EUS-diseased snakehead fish in the canal at about the same time. However, the farm manager at site 2 did not cut off the water supply on the first day and a few days later, some diseased fish appeared in many ponds. The water supply was then cut off and lime was applied but fish in all 40 ponds were soon infected. The percent affected fish in each pond varied between 5%-80% (Khun Somkid, farm manager, pers. comm.). Samples were taken on 8th February 1994 for virus isolation studies.

3.3.3 Late Period EUS

Late period EUS fish samples were collected on 28th February 1994, 6-8 weeks after the outbreak started. The collection site and samples were as follows:

- **Prachinburi :** This fish farm had a polyculture and semi-intensive culture system (medium stocking density and feed was given). There were 15 ponds varying in size from 18 to 30 rai. Many fish species were raised including striped snakehead,

snakeskin gourami (*Trichogaster pectoralis*), climbing perch (*Anabas testudineus*), common carp, nile tilapia, and silver barb. The water supply was pumped directly from Prachinburi river to the water-inlet canal then to the fish ponds. EUS outbreaks have occurred annually in this fish farm since the early 1980s'. The farm experienced mass mortalities of fishes due to EUS in the mid I980s but in later years outbreaks caused only a few percent losses. In 1994, the outbreak occurred in January and affected striped snakehead and snakeskin gourami with an estimated 5-10% loss. The rest of the cultured species were unaffected. There were also low numbers of a few fish species which had entered the ponds via the water supply. These included swamp eel (*Fluta alba*), spiny eel (*Mastacembelus* spp.), three-spot gourami and walking catfish (*Clarias batrachus*) which were also reported to be affected by EUS (Kuhn Santipab, farm owner and manager, pers. comm.). Unfortunately, by the time of sampling, only 2 striped snakehead fish with EUS lesions were available from the farm and brought back to AAHRI for virus isolation studies.

3.3.4 Recovery Period EUS

Recovery period EUS samples were collected from Suphanburi site 1 and Bangkok 4-5 weeks after the disease had apparently disappeared.

The samples from Suphanburi site 1 were collected on 1st April 1994 at the time of fish harvesting. The farm manager estimated that 80% of the fish in the pond had been lost during the outbreak. Among the survivors, over 80% were found to have some external signs remaining from the disease such as scar tissue or healed wounds on the trunks and

heads. Twenty-two fish (averaging 363.3 g) were randomly collected from these surviving fish for virus isolation. Blood samples were collected from caudal blood vessels using syringes and needles and the sera retained at -70°C for further studies (Chapter 6).

The samples from Bangkok were collected on 5th April 1994. The farmer estimated that 40-50% of snakehead fish in the rice paddy field had been lost during the outbreak. Less than 50% of the survivors showed healed wounds on their trunks and bodies. The farmer kindly contributed 5 snakehead fish (averaging 30 g) for virus isolation.

The live specimens were collected and held in a tank containing cooled pond water (20-25°C) and transported in an air-conditioned mini-truck to the Virology Unit at the Aquatic Animal Health Research Institute (AAHRI) in Bangkok. Transportation time from each location was less than 2 h. Upon arrival, tissue extracts were prepared for virus isolation. The sample codes, location and disease status are given in Table 3.1.

Table 3.1 EUS-diseased samples collected at different periods of the epizootic in 1993/1994 in Thailand.

EUS condition	Coding	Location	Type of fish culture	Disease onset	Sampling date and time	Temperature on sampling date (water/air °C)	Fish species and weight
Early period EUS	AV9402	Suphanburi (site 1)	Intensive (monoculture)	30/12/93	4/1/94 11.00 am	26/29	6 striped snakehead fish (*Channa striatus*) (~250 g/fish)
	AV9404	Bangkok	Extensive (polyculture in rice paddy field)	1/1/94	7/1/94 11.00 am	26/29	6 striped snakehead (~25 g/fish) 3 three-spot gourami (*Trichogaster trichopterus*) (~8 g/fish)
	AV9405	Suphanburi (site 1)	Intensive (monoculture)	30/12/93	11/1/94 11.00 am	27/30	7 striped snakehead fish (~250 g/fish)
Mid period EUS	AV9408	Suphanburi (site 2)	Intensive (monoculture)	30/12/93	8/2/94 12.00 am	29/32	6 s striped nakehead fish (~300 g/fish)
Late period EUS	AV9412	Prachinburi	Semi-intensive (polyculture)	Mid January 1994	28/2/94 11.00 am	29/33	2 striped snakehead fish (~300 g/fish)
Recovery period EUS	AV9420	Suphanburi (site 1)	Intensive (monoculture)	30/12/93	1/4/94 12.00 am	30/33	22 striped snakehead fish (~360 g/fish)
	AV9421	Bangkok	Extensive (polyculture in rice paddy field)	1/1/94	5/4/94 11.00 am	29/33	5 striped snakehead fish (~30 g/fish)

3.4 Results

All diseased snakehead fish showed classic clinical signs of EUS. Affected fish had at least one medium to large lesion on the body. Ulcers were shallow in early period EUS and deeper in middle period and late period EUS. The shallow ulcers were pink to red in colour and covered with a light-brown filamentous mat consisting of fungal hyphae, many kinds of microorganism and colloidal particles of soil. Fish showed reduced movement and stayed near the water surface. Swimming with the head out of the water was often observed in fish with head lesions. Some fish had lost their eyes and exhibited a darker body colour. Some fish showed head erosion and exposure of anterior brain to the water. Deep ulcerative lesions were often found in late period EUS with exposure of part of the bony skeleton and/or internal organs.

Three-spot gouramis collected during early period EUS showed red, haemorrhagic lesions around the vent, anal fin and caudal peduncle. Snakehead fish recovering from EUS remained with deformities of the head. Ulcers had healed and scales were restored. Illustrations of the sampling sites and diseased fishes are shown in Figures 3.1-3.16.

3.4.1 Early Period EUS

Sample code AV9402

The 6 striped snakehead specimens collected from Suphanburi site 1 on 4th January 1994 died during transportation to the laboratory at AAHRI. Upon arrival, tissues were

Figure 3.1 Suphanburi site 1: 40 ponds in the farm. Pond sides are lined with concrete blocks. Area of each pond is 1.5 rai (1 rai = 1600 m^2). This farm has an intensive monoculture culture system with continuous flow through water supply. Suphanburi site 1 and site 2 have the same pond design and water supply. EUS was first seen in the irrigation canal on 30th December 1993 and spread into culture ponds.

Figure 3.2 Bangkok site: A rice paddy field has an area of 18.5 rai. It was a polyculture with extensive culture system. The water was drained in the middle of December and the fishes gathered in 1 pond size 6 × 100 × 2.5 m^3 (W×L×H). EUS initially occurred on 1st January 1994.

Figure 3.3 Prachinburi site: 15 ponds varying in size between 18 and 30 rai/pond. This farm has polyculture and semi-intensive culture system. EUS was first seen in the river and spread into culture ponds about middle of January 1994.

Figure 3.4 Illustration of sample collection method in diseased pond of Suphanburi site 1. Only a small hand-net was allowed to catch the diseased fish and the samples were collected on 11th January 1994. A large number of dead fish was observed and the pond water deteriorated rapidly with an unfavorable smell. Arrow indicated a bamboo raft for feeding purposes.

Figure 3.5 Diseased fish with severe head injury exhibiting a swimming motion with head out of the water. Many affected fish were slow moving and stayed near the water surface.

Figure 3.6 Fish suffering from head and brain damage and loss of both eyes. The blind fish exhibited dark coloration and a swimming motion with head out of the water.

Figure 3.7 Severe head erosion observed within 1 week of onset of disease. The maxillary jaw and anterior part of the skull were eroded away. Eyeballs also severely infected. This fish collected at Suphanburi site 1 on 4th January 1994.

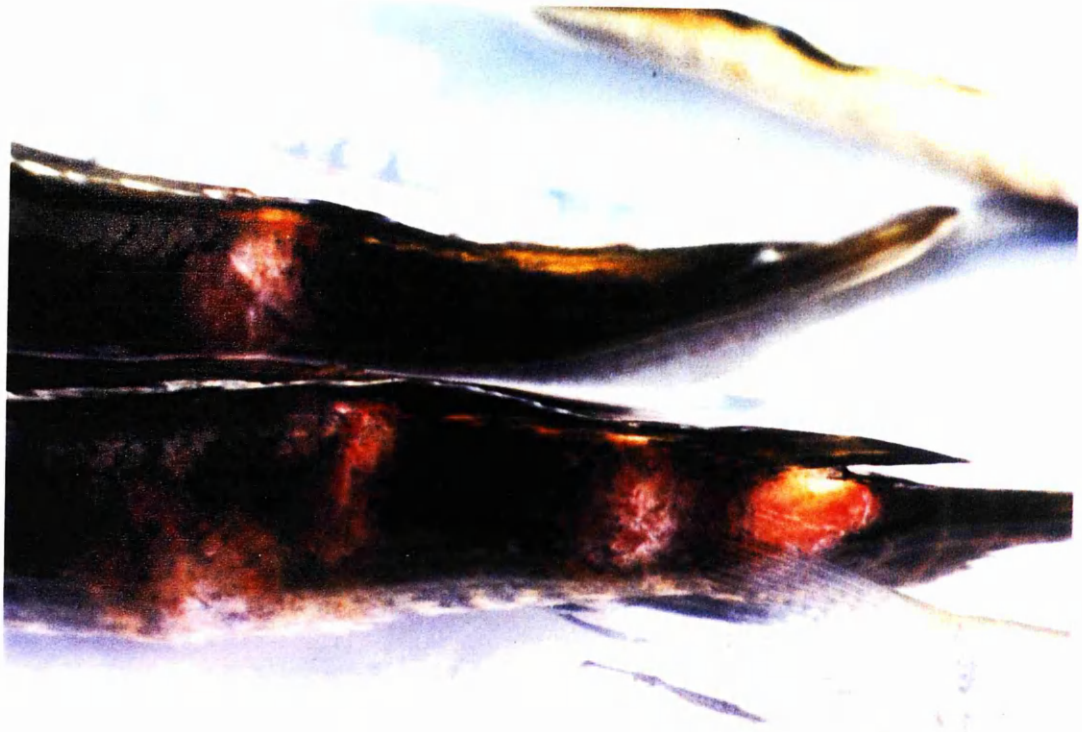
Figure 3.8 Classic EUS lesions of striped snakehead fish. Multiple shallow ulcers were pink to red in colour and covered with light-brown filamentous material. This fish collected at Suphanburi site 1 during the early period of the outbreak.

Figure 3.9 Snakehead fish from Suphanburi site 1 dead from EUS. Fish usually had multiple ulcerative lesions. Losses up to 50% on day 5 and up to 70% on day 12 were recorded.

Figure 3.10 EUS-affected fish from Bangkok showing lesions on anterior part of the body. The cornea of 1 eye had turned white. The ulcerative lesion was covered with fungal hyphae. The fish were collected on day 7 after the outbreak started.

Figure 3.11 Snakehead fish with classic EUS lesions on the posterior part of the body collected from Bangkok on day 7 after the outbreak started. The multiple lesions observed were pink to red in colour and covered with fungal hyphae. No deep ulcerative wounds were seen in juvenile fish.

Figure 3.12 Early signs of the EUS in three-spot gourami fish from Bangkok site. The fish exhibited haemorrhagic lesions around the posterior and ventral part of their bodies, pelvic fin, vent, base of anal fin and caudal peduncle.



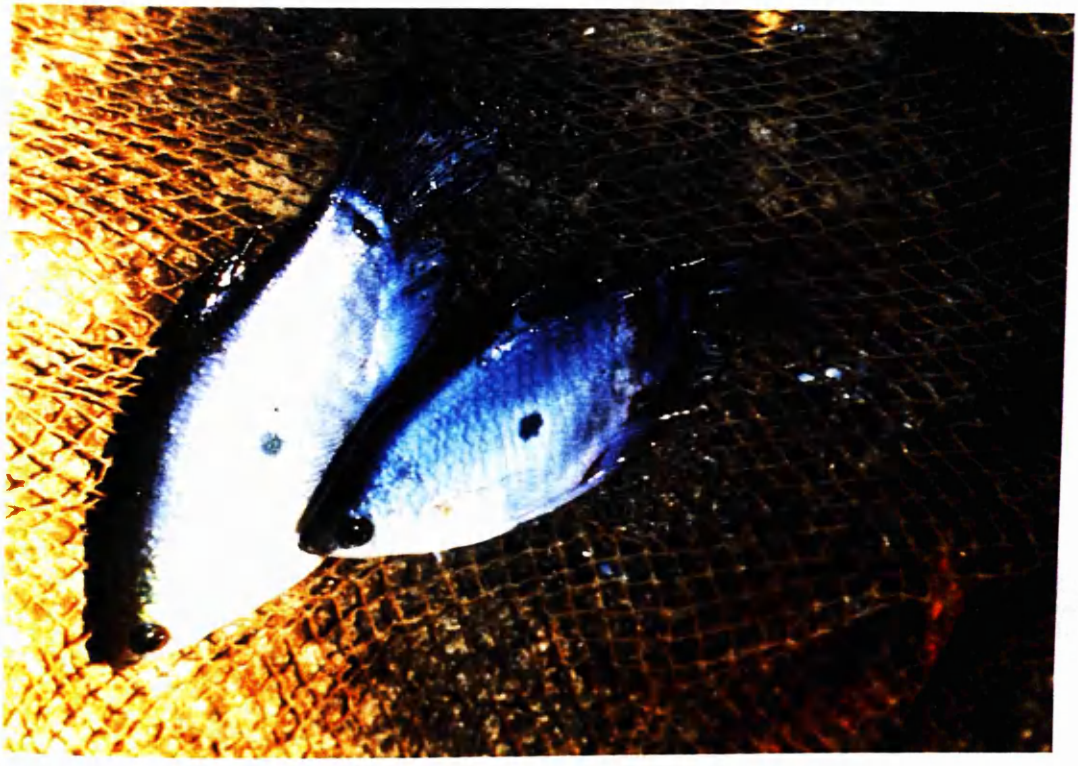


Figure 3.13 Middle period EUS fish collected from Suphanburi site 2 on 28th February 1994. Most affected fish in the pond had head lesions. Some fish had no lesion or few lesions on the flank. Fish with multiple ulcerative lesions were not seen or might have died.

Figure 3.14 Late period EUS fish collected from Prachinburi province on 28th February 1994. They showed a large open wound on the abdomen. The internal organs were exposed to the water.

Figure 3.15 Late period EUS lesion from Prachinburi province showing a large open wound on dorsal region close to the head. The muscle was destroyed deep into the body.

Figure 3.16 Recovered snakehead fish collected from Suphanburi site 1 on 1st April 1994. The lesions had healed and the scales restored. Only 20% of the fish survived the disease outbreak but 80% of the survivors remained with a disfigured head and could not be sold as fresh fish in the market.

immediately processed for virus isolation. Muscle tissue from 2 fish was pooled as 1 sample and the internal organ tissues pooled as a separate sample. The total of 6 samples of filtered tissue extract was held at 4°C for 3 days before simultaneous inoculation into HCK, BF-2, SSN-1 and EPC cells in 24-well plates. Twelve wells of each plate were assigned for the samples (2 wells/sample). The other 12 wells were reserved for the five AV9404 samples and control cells (Table 3.2).

All 4 cell lines showed minor cytopathic changes on days 3-5 after inoculation. Although the cells seemed to round-up, these changes did not progress and a more positive CPE did not develop with further incubation. A first passage of culture fluids was performed for all cell lines. No transmissible CPE was obtained. Sample-inoculated cells were normal when compared to control cells. No cytopathic changes were observed in the second passage.

No virus was isolated or positive CPE obtained from the freshly dead fish specimens collected from Suphanburi site 1 during the early period EUS.

Sample code AV9404

The 6 striped snakehead and 3 three-spot gourami fish with classic EUS collected from the Bangkok site were still alive on arrival at AAHRI. Muscle tissue from 3 snakehead fish was pooled as 1 sample and the internal organ tissues pooled as a separate sample. A single sample was prepared from the 3 gouramis, consisting of a pool of both internal organ and muscle tissues. All samples were inoculated on the day of collection on to the same 4 cell lines in the same 24-well plates as AV9402 (Table 3.2).

Following the initial inoculation of samples, HCK cells gave 3 positive CPE cultures and BF-2 gave 4 positive CPE cultures out of the 5 samples. There was a minor cytopathic change on day 7 in the HCK cells inoculated with the gourami sample but the change did not progress to a positive CPE. The cells recovered and remained as a confluent monolayer. The first and second passages of the gourami sample were also CPE negative. Snakehead sample 7 showed no CPE in the initial inoculation in every cell line. However, a positive CPE with sample 7 was obtained from the first passage in HCK, BF-2 and SSN-1 cell cultures.

The SSN-1 and EPC cell lines both showed 1 negative CPE (sample 7) and 4 doubtful CPE samples from the initial inoculation. At the first passage, SSN-1 cells gave a positive CPE for all 5 samples, but the EPC line revealed only 2 doubtful CPE samples. The EPC cells showed a rounding-up type CPE involving over 50% of the monolayer on days 3-5 for snakehead samples 8 and 9 which gradually decreased and stopped on days 7-9 when the cells started to regenerate and were restored to confluency. The second passage performed on day 7 gave no CPE. No suspected virus was therefore isolated in EPC cells.

The finding of a positive CPE which could be transmitted to the next passage in 3 cell lines (Figure 3.17) indicated successful virus isolations from the 5 extracts of pooled tissue samples from 9 fish for which 4 isolates were obtained from the 6 live snakehead fish and the fifth isolate from the three-spot gouramis. Five virus isolates were given

new codes T9412, T9413, T9414, T9415 and T9416 as shown in Table 3.2. All isolates were later identified as rhabdoviruses (Chapter 6).

Table 3.2 Observations of CPE in cell cultures from EUS sample code AV9402 and AV9404. Tests were conducted in 24-well plates. The supernatants (1:50 dilution) of tissue extract were simultaneously inoculated onto 4 cell lines. Two replicate wells were set up for each sample and control. The cells were incubated at 23°C. The first passage was performed on day 10.

Cell line	Culture passage	AV9402 6 dead EUS fish						AV9404 9 live EUS fish					
		1	2	3	4	5	6	7	8	9	10	11	12
		mus	mus	mus	int	int	int	mus	mus	int	int	mus + int	con
HCK	ini	?	?	?	?	?	?	–	+	+	+	?	–
	1 st	–	–	–	–	–	–	+	+	+	+	–	–
	2 nd	–	–	–	–	–	–	+	+	+	+	–	–
	3 rd	nd	nd	nd	nd	nd	nd	+	+	+	+	nd	–
BF2	ini	?	?	?	?	?	?	–	+	+	+	+	–
	1 st	–	–	–	–	–	–	+	+	+	+	+	–
	2 nd	–	–	–	–	–	–	+	+	+	+	+	–
	3 rd	nd	nd	nd	nd	nd	nd	+	+	nd	nd	+	–
SSN1	ini	?	?	?	?	?	?	–	?	?	?	?	–
	1 st	–	–	–	–	–	–	+	+	+	+	+	–
	2 nd	–	–	–	–	–	–	+	+	+	+	+	–
	3 rd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	–
EPC	ini	–	–	–	?	?	?	–	?	?	?	?	–
	1 st	–	–	–	–	–	–	–	?	?	–	–	–
	2 nd	–	–	–	–	–	–	–	–	–	–	–	–
virus code		–	–	–	–	–	–	T9412	T9413	T9414	T9415	T9416	–

Samples 1-6; pools from 2 snakehead fish
Samples 7-10; pools from 3 snakehead fish
Sample 11; pool from 3 three-spot gourami fish.

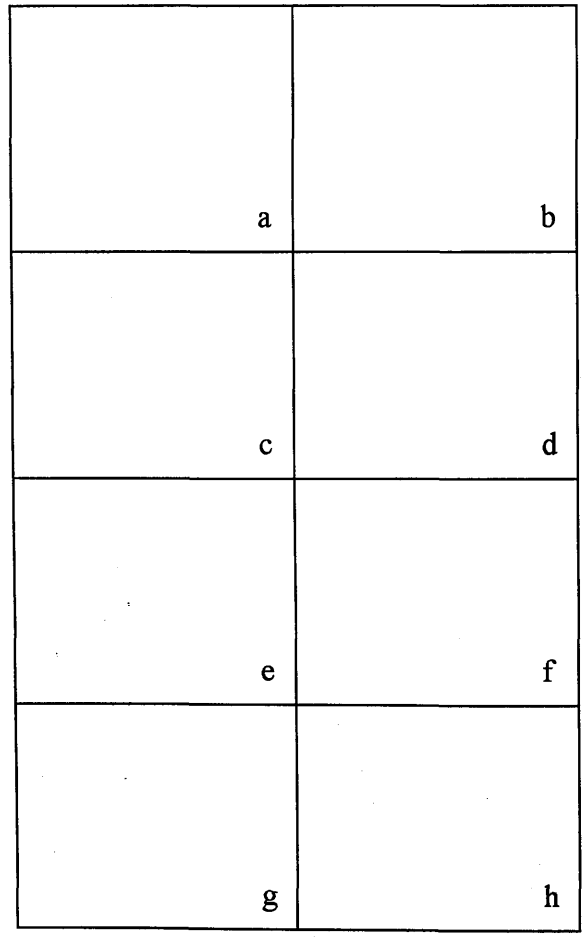
ini = initial inoculation
1 st = first passage
mus = muscle tissue sample
int = internal organ tissue sample
con = control
nd = not done

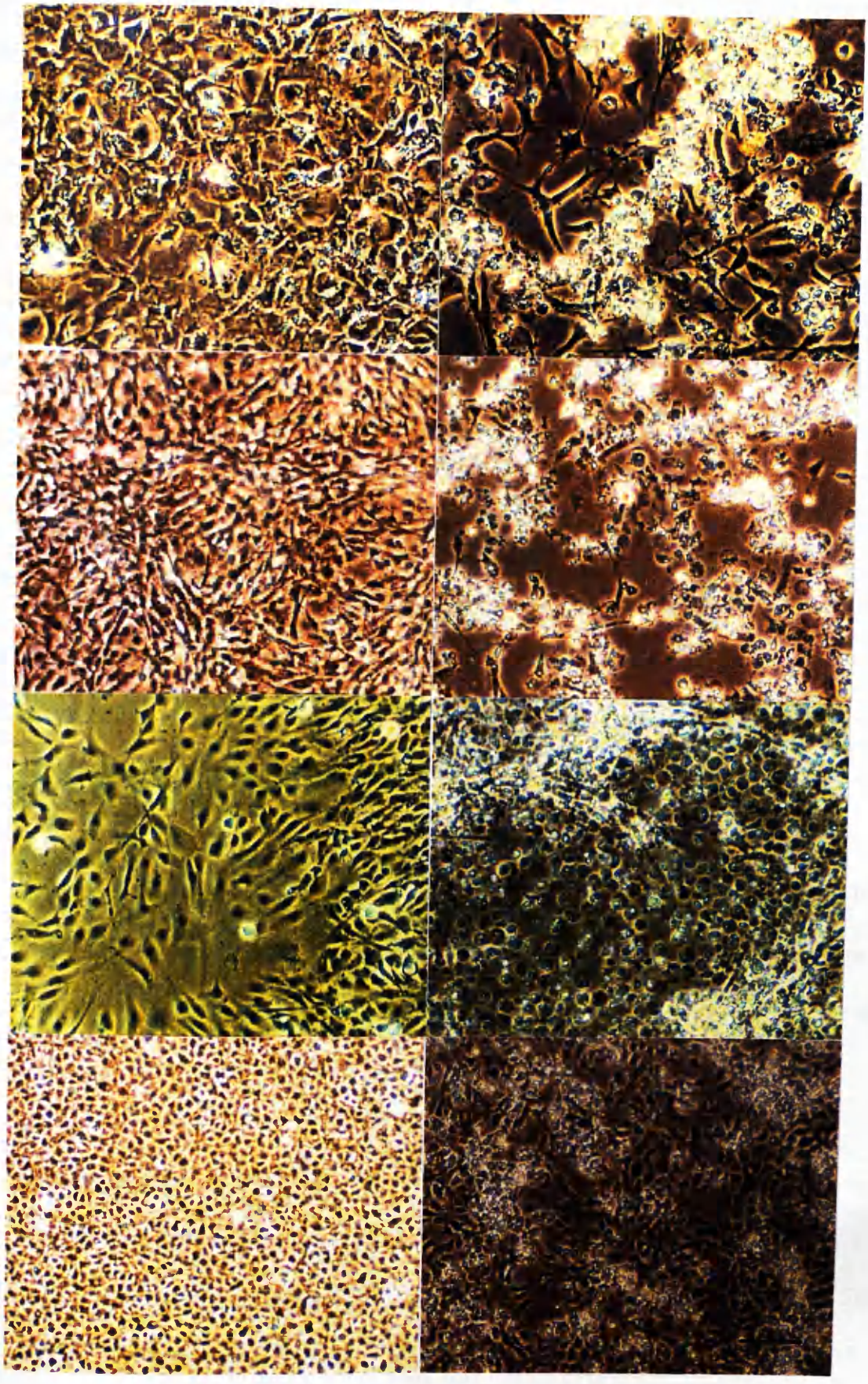
– = negative CPE
? = doubtful CPE
+ = positive CPE

Figure 3.17 Cytopathic effects on 4 cell lines caused by the inocula prepared from EUS-diseased fishes. Bar = 110 μm

a. control HCK on day 7; b. HCK with 1st passage of sample 10 on day 7;
c. control BF-2 on day 5; d. BF-2 with 3rd passage of sample 11 on day 5;
e. control SSN-1 on day 3; f. SSN-1 with 3rd passage of sample 8 on day 3;
g. control EPC on day 3; h. EPC with 1st passage of sample 8 on day 3.

The CPE on the EPC cells gradually disappeared. The cells returned to normal and restored a confluent monolayer.





Sample code AV9405

Seven EUS-infected striped snakehead samples were collected from the same pond as AV9402 (Suphanburi site 1) 1 week after those of AV9402. This second set of samples was also classified as early period EUS. Five fish died in transit but the other 2 were alive on arrival at the laboratory. Tissue samples were processed and inoculated on the day of arrival. Cell lines used were the same as for AV9402 and AV9404. As shown in Table 3.3, samples 1-7 were from the 5 dead fish while samples 8-11 were from the 2 live fish.

After initial inoculation, BF-2, SSN-1 and EPC cells appeared to develop more marked cytopathic changes than HCK cells. Both groups of fish samples (dead and live fish) caused some cytopathic changes in all cell cultures. Three samples gave a positive CPE in BF-2 cells, 2 samples a positive CPE in EPC cells and 1 sample a positive CPE in SSN-1 cells. Many doubtful CPE samples were also observed.

On first passage, some initially positive samples (e.g. sample 2 in BF-2, SSN-1 and EPC cells) did not transmit a CPE while some doubtful CPE (samples 8-9) turned to positive CPE as shown in Table 3.3. The HCK and EPC cells gave a negative CPE for all samples on both first and second passage.

At the second and third passages BF-2 was found to be one of the most sensitive cells. Four positive CPE samples were obtained in BF-2 cells. Three of these (samples 9, 10 and 11) were obtained from the 2 live fish, while the other was obtained from 1 dead fish (sample 5). The second passage of samples 9-10 in SSN-1 cells yielded a continuing

CPE. Four suspect viruses were thus isolated from the specimens collected from Suphanburi site 1 (AV9405) during the early period of the EUS outbreak and coded as isolates T9424, T9428, T9429 and T9430 as shown in Table 3.3. All suspect viruses were rhabdoviruses (Chapter 6). It was noted that the viruses were more likely to be isolated from live fish than dead fish.

Table 3.3 Observations of CPE in cell cultures from EUS sample code AV9405. Tests were conducted in 24-well plates. The supernatants (1:50 dilution) of tissue extract were simultaneously inoculated onto 4 cell lines. Two replicate wells were set up for each sample and control. The cells were incubated at 23°C. The first passage was performed on day 10.

Cell line	Culture passage	AV9405											
		5 dead EUS fish							2 live EUS fish				
		1	2	3	4	5	6	7	8	9	10	11	12
		mus	int	mus	int	int	mus	int	mus	int	mus	int	con
HCK	ini	–	?	–	–	–	–	–	?	–	–	–	–
	1 st	–	–	–	–	–	–	–	–	–	–	–	–
	2 nd	–	–	–	–	–	–	–	–	–	–	–	–
BF2	ini	?	+	–	?	?	?	?	+	+	?	?	–
	1 st	–	–	–	–	–	–	–	–	+	–	–	–
	2 nd	–	–	–	–	+	–	–	–	+	+	+	–
	3 rd	nd	nd	nd	nd	+	nd	nd	nd	+	+	+	–
SSN1	ini	–	+	–	?	–	–	?	?	?	?	?	–
	1 st	–	–	–	–	–	–	–	–	+	+	–	–
	2 nd	–	–	–	–	–	–	–	–	+	+	–	–
EPC	ini	?	+	–	–	–	–	–	+	?	?	–	–
	1 st	?	–	–	–	–	–	–	–	–	–	–	–
	2 nd	?	–	–	–	–	–	–	–	–	–	–	–
virus	**code**	–	–	–	–	T9424	–	–	–	T9428	T9429	T9430	–

Samples 1-3; pools from 2 EUS fish
Samples 4-11; prepared from individual EUS fish
ini = initial inoculation
1 st = first passage
mus = muscle tissue sample
int = internal organ tissue sample
con = control
nd = not done

– = negative CPE
? = doubtful CPE
+ = positive CPE

3.4.2 Middle Period EUS

Sample code AV9408

Twenty-two classic EUS-infected striped snakehead fish were collected from Suphanburi site 2 in the middle period EUS. Most fish samples had large ulcerative lesions on the head. Fifteen fish died during transportation. Six live specimens were used for virus isolation. Six muscle tissue and 6 internal organ tissue samples were prepared. Cell line indicators were BF-2 and SSN-1. One live snakehead fish with no caudal peduncle and tail was kept alive in an aquarium. Eventually, 3 days later it died.

Following initial inoculation on BF-2 cells, all 12 samples caused minor cytopathic changes with rounding-up of cells on days 5-10. These changes did not progress to show a positive CPE and the cells returned to a normal appearance after day 10. The first and second passages revealed a negative CPE. With SSN-1 cells, the initial inoculation, first and second passages of the 12 samples all gave a negative CPE.

No suspect virus was therefore isolated from the specimens collected during the middle period of the EUS outbreak.

3.4.3 Late Period EUS

Sample code AV9412

Two classic EUS fish specimens were obtained from Prachinburi province during the late period of the EUS outbreak. The fish had large ulcerative lesions on the body and exposed internal organs. As they were virtually moribund it was decided to transport

them in an ice-cooled styrofoam box. Upon arrival, 2 muscle tissue and 2 internal organ tissue samples were prepared for virus isolation. Cell line indicators were BF-2 and SSN-1.

There was no cytopathic changes on either cell line after initial inoculation, first or second passage. Therefore, no suspect virus could be isolated from the specimens collected during the late period of the EUS outbreak.

3.4.4 Recovery Period EUS

Sample code AV9420

Twenty-two EUS-recovered striped snakehead fish were collected from the same pond as sample codes AV9402 and AV9405 (Suphanburi site 1). Sampling took place 4-5 weeks after the end of the EUS outbreak. The fish were alive on arrival at the laboratory. The internal organs were selected for preparation for virus isolation. The organs from each of 2 fish were pooled and numbered as 1 sample. Eleven samples of tissue extract were prepared for inoculation. Cell line indicators were HCK and BF-2.

The initial inoculation, first and second passages gave clearly negative CPE. Therefore, no suspect virus could be isolated from the EUS-recovered fish collected from Suphanburi site 1.

Sample code AV9421

Five striped snakehead fish were collected from the same Bangkok pond as sample code AV9404. Sampling was carried out 4-5 weeks after the end of EUS outbreak. Two fish had scar tissue or healed lesions on their bodies. Three appeared as normal fish. Internal organs and muscle tissue from 1 fish were pooled and numbered as 1 sample. Five samples of tissue extract were prepared for inoculation. Cell line indicators were HCK and BF-2.

The initial inoculation, first and second passages showed clearly negative CPE. No suspect virus was therefore isolated from the EUS-recovered fish collected from Bangkok.

3.5 Discussion

The EUS outbreaks in 1993-1994 did not spread as widely as in previous years. However, a severe clinical condition was still manifested in affected fish with moderate to large ulcerative lesions developing on the body and head. The massive and rapid deaths of fish in the diseased ponds were very similar to the outbreaks of the early years. Initial outbreaks can occur in wild or farmed fishes. A good record of fish losses was obtained from the farm manager of Suphanburi site 1. Fish density in the pond during the outbreak was ~15 fish/m^2 or ~33000 fish/pond. An estimated 50% loss occurred by day 5 and 70% loss by day 12 after the onset of the disease. At harvesting, only 20% fish remained and most (80%) of them exhibited scar formation particularly on the head.

It was noted that the outbreaks at Suphanburi site 1 and site 2 initially occurred in the natural water supply. Natural water may carry a variety of pathogenic agents, possibly viruses and/or fungi, which may be present in very much higher numbers than in pond water. On the other hand, the condition of the natural water may provide more support to the causative agents of EUS than the water conditions in the intensive fish culture ponds which have high levels of organic matter and/or micro-organism, including plankton. The 2 immediate actions of cutting off the water supply and liming the pond were well proven as preventative measures. These actions may directly reduce or kill a number of pathogens in the pond or may create changes in the pond ecosystem that may generate some forms of inhibition against the causative agents as intensive snakehead fish ponds usually have high organic loads and nitrogenous waste levels from the trash fish food that pollute the pond soon after the water supply is cut off. Multiple ulcerative lesions were common on snakehead fish during early period of the outbreak and these fish might die rapidly. Only affected fish with 1 or 2 ulcerative lesions were likely to survive longer and had a greater chance of recovery.

As a result of virus isolation studies, 9 isolates were successfully recovered in the laboratory. All 9 isolates belonged to a single virus family, Rhabdoviridae (Chapter 6). This is the second occasion in which a single virus family has been isolated from different fishes and farm locations in Thailand. In the first instance, Frerichs *et al.* (1989b) isolated rhabdoviruses from EUS-infected samples of Southeast Asia and Sri Lanka. The results from the present study together with those of Frerichs and colleagues indicate a more important role for a rhabdovirus than the variety of virus-like particles (birnavirus, picornavirus, arenavirus, bunyavirus, myxovirus and unidentified viruses)

seen in electronmicrographs (Wattanavijarn *et al.*, 1985), the EUS-associated reovirus (Roberts *et al.*, 1994) and 4 EUS-associated birnaviruses (Hedrick *et al.*, 1986; Saitanu *et al.*, 1986; Subramaniam *et al.*, 1993; Wattanavijarn *et al.*, 1988). However, a rhabdovirus, birnavirus or reovirus is unlikely to have sole responsibility as the infectious agent for over 100 fish species in Asian countries which have been recorded as susceptible to EUS (Lilley *et al.*, 1992). All viruses may only comprise one part of a complex of etiological agents.

It was noted that rhabdovirus was isolated only from samples collected during the first 2 weeks of the EUS outbreak. Virus isolation procedures at subsequent periods (middle, late and recovery) gave clearly negative results in the indicator cell lines. There was not even 1 doubtful CPE from the 31 fish samples examined. This finding may be very significant in considering how difficult it is to isolate viruses from EUS-diseased fish. It has been believed since the early 1980s' that some type of infectious pathogen must be involved as a primary causative agent, and a rhabdovirus has been nominated among them (Roberts *et al.*, 1986; Frerichs *et al.*, 1986; Frerichs *et al.*, 1989b). The uncertainty regarding the role of a virus as a primary causative agent arose from the low frequency of virus isolation from EUS-diseased fishes collected from southeast Asia and the fact that no virus was isolated from a large number of diseased fish samples from the major EUS outbreak in Bangladesh (Roberts *et al.*, 1994; Frerichs, 1995). With reference to the results of this present study, the low overall frequency of virus isolation may have been due to sampling after the early period of the outbreak, as no viruses were isolated from samples collected 5 weeks after the start of the epizootic. These findings are in general agreement with virus isolation attempts from other rhabdovirus diseases of fish

such as VHSV, which was easily isolated from diseased fish during an epizootic but not from the surviving fish (Wolf, 1988). For IHNV epizootics in salmonid hatcheries, virus usually caused 80-90% death in fish but could be isolated from the survivors only within 7 weeks after the onset of disease (Leong, 1995). The amount of isolatable viruses in the diseased fish decreased after the start of the outbreak and similar events were also demonstrated in experimental infections with IHNV (Bootland *et al.*, 1994), SVCV (Ahne, 1986) and IPNV (Frantsi and Savan, 1971). However, the onset and duration of EUS outbreaks may vary from year to year and from country to country and are likely to be directly related to the duration of cool seasons. The period of successful virus isolation may therefore also vary from one outbreak to another.

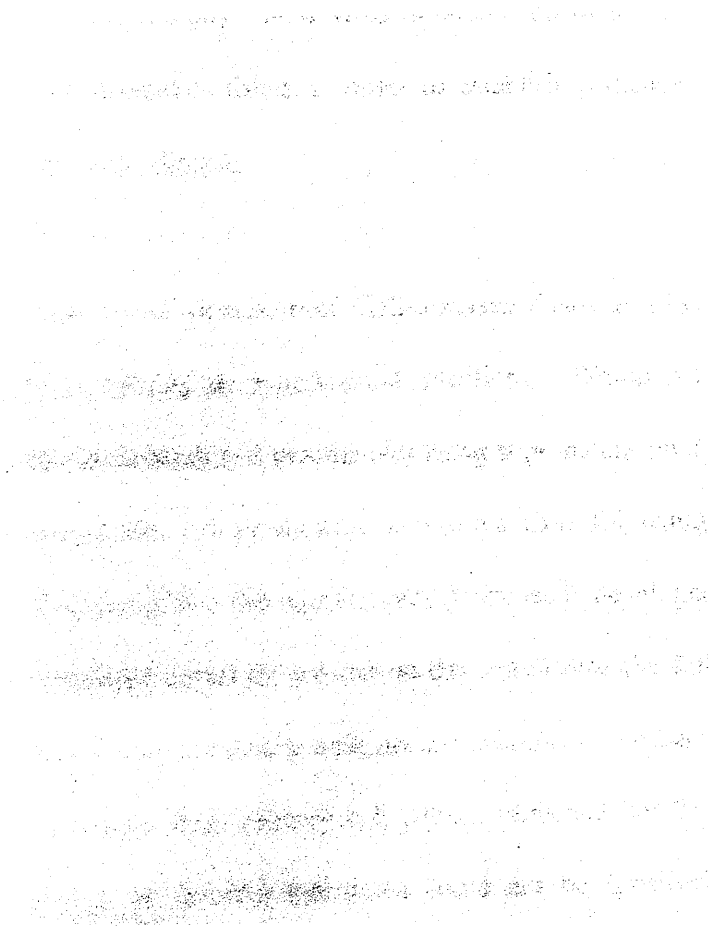
Furthermore, fish had to be alive immediately before sampling to achieve the best virus isolation result. This was apparent from samples AV9402 and AV9405 collected from the same ponds in Suphanburi province. Thirteen tissue extracts prepared from 11 dead snakehead fish gave only 1 virus isolate (< 8%), while 4 tissue extracts from 2 live snakehead fish provided 3 virus isolates (75%). Additionally, 5 tissue extracts giving 100% successful virus isolation came from 9 live fish in Bangkok sample AV9404. This finding agrees with the suggested general technique for specimen collection by Hetrick (1989).

This observation does not indicate how long the specimen could be kept in transport medium or when tissue extracts should be inoculated on to cells following preparation but suggests that live specimens are preferable and should be processed and inoculated on the day of collection. Hetrick (1989) suggested the tissue specimens can be held in

transport medium on ice or in a refrigerator only for several hours before processing but if the specimen needs to be kept longer, it is preferable to store frozen at -80°C. However, for EUS-diseased fish, it is possible to isolate birnavirus from the fresh specimen directly stored at -20°C for more than 1 year (Saitanu *et al.*, 1986). The success of rhabdovirus isolation from internal organs and from muscle tissue beneath the ulcer of EUS-affected fish indicates that the virus causes a generalised viremic infection which is a common characteristic of pathogenic fish rhabdoviruses (Ahne *et al.*, 1988). Samples from internal organs are likely to give better virus recovery. Five isolates were obtained from internal organs (kidney, spleen, pancreas, small intestine and liver), while 3 isolates were obtained from muscle tissue.

Among the 4 cell lines used, BF-2 seemed to be the best followed by SSN-1 and HCK lines. EPC cells seemed to develop some changes due to the suspect viruses but this did not progress to cause complete CPE. Frerichs *et al.* (1989b) found SSN-1 to be more sensitive for virus isolation than BF-2, RTG-2 or EPC lines. Interestingly, BF-2 cells were also used to isolate a birnavirus, SGV, from EUS-diseased sand goby fish in cage culture in central Thailand (Hedrick *et al.*, 1986) but the same cell line used for virus isolation in this study isolated only rhabdoviruses from diseased snakehead fish and three-spot gouramis in the same geographical area. The recovery different viruses indicates that each virus may infect a selected group of fish species. Other established lines such as BB, FHM, CHSE-214 have been successfully used to isolate birnavirus from EUS outbreaks (Hedrick *et al.*, 1986; Saitanu *et al.*, 1986). It was therefore of interest to test the sensitivities of the present rhabdovirus isolates on these 3 cell lines (Chapter 6).

The recovery of the rhabdovirus in the early period of the EUS outbreak indicates that the rhabdovirus is likely to be one of the complex of etiological agents.



Chapter 4

Experimental Infection Using a New Rhabdovirus Isolate

4.1 Introduction and Literature Review

One of the most important steps following successful virus isolation from diseased fish is an experimental infection study. New virus isolates need to be examined in the same host fish, or other susceptible fishes, in order to establish virulence, pathogenicity and causal relationship to the disease.

Since the first recovery of viruses from EUS-diseased fishes in 1985, there have been only a few reports relating to experimental infections. Wattanavijarn *et al.* (1985) infected 5 juvenile snakeheads (~2 months old) using supernatant fluid from primary cell culture of snakehead fish kidney showing a positive CPE following inoculation with tissue extract. Two out of five fish injected intra-peritoneally developed a lesion near the operculum and exhibited dorsal fin erosion on day 2 and died the following day. Five control fish injected intraperitoneally with normal saline and 5 other fish injected with normal supernatant fluid from primary cell culture remained healthy and suffered no mortality. The type of virus(es) inoculated could not be specified by the authors. However, when the cell debris of the primary cell cultures was pelleted, processed and examined by transmission electron microscopy, rhabdovirus-like and IPNV-like particles were found.

A further experimental infection study was reported by Saitanu *et al.* (1986). An EUS-isolated virus, SHV-1, passages 3 and 4 in BB cells was used to inoculate 2 sizes of snakehead fish. The water temperatures were 25-28°C. It was noted that 80% (32/40) of virus-injected juvenile snakehead fish, 10-15 cm or 30-80 g, developed scale erosions and shallow ulcers. There were no clinical changes in virus-injected young snakehead fish, 21-26 cm (or 100-250 g), and no mortality of either fish size during 14 days observation. These findings seem to suggest that the virulence of SHV-1 virus is related to the age of the fish.

More recent experimental infection work was reported by Frerichs *et al.* (1993). Infectivity experiments were performed in 3 months old juvenile snakehead fish using an EUS-isolated rhabdovirus and cell line-associated retrovirus. The latter virus was obtained from supernatant fluid of normal snakehead fish fry cell line (SSN-1) (Frerichs *et al.*, 1991). Experimentally infected fish were held in glass aquaria containing pond water or underground water at 20-25°C. The pond water was brought from an EUS affected fish pond during a disease outbreak. During a 1 month observation period, no ulcerative lesion developed in any experimental fish, but some rhabdovirus-infected fish showed superficial skin damage (Frerichs *et al.*, 1993).

The above 3 reports showed that the EUS-associated viruses could cause only minor clinical effects in experimentally infected fish with no deep ulcerative lesions being demonstrated. The virus seems to cause more damage in younger fish. The effect of temperature on virus virulence could not be predicted or compared from these 3 reports.

However, temperature, age of fish and route of challenge are important factors related to the virulence and pathogenicity of other fish viruses.

Water temperature has been shown to have a marked effect to the virulence of fish viruses. Under experimental conditions, viral haemorrhagic septicaemia (VHS) virus caused disease and mortalities in sea bass (*Dicentrachus labrax*) and turbot (*Scophthalmus maximus*) at 12-14°C, but did not give rise to any signs of disease in these fishes at 20°C (Castric and de Kinkelin, 1984). Similar findings of lower mortality of infected fishes at higher temperatures have also been reported for infectious pancreatic necrosis virus (IPNV) (Frantsi and Savan, 1971), infectious hematopoietic necrosis virus (IHNV) (Amend, 1970) and spring viremia of carp virus (SVCV) (Ahne, 1980). An attempt to link seasonal variation with virulence of a fish virus was reported by Baudouy *et al.* (1980) who performed a 210 days experimental infection of carp fingerlings with SVCV. It was found that SVC virus caused low mortalities during the autumn-winter season whereas massive mortalities were found during the winter-spring season just after the water temperature rose above 7°C.

The age of fish is another factor related to the virulence of fish viruses. There are a number of reports in which older fish have shown more resistance to virus infection than younger fish. Frantsi and Savan (1971) infected brook trout at different ages with IPN virus at 10°C. Mortalities up to 83% were observed in fish aged 1 month but values less than 75%, 50% and 5% were recorded in fish aged 2, 4 and 6 months, respectively. Bootsma *et al.* (1975) conducted an experimental infection of pike eggs and pike fry with pike fry rhabdovirus (PFRV). Fertilised eggs were bathed with PFRV for 3 h.

After hatching, mortalities started to appear on day 14 and all fry died by day 21. Fry (4-5 cm in length) bathed with a similar amount of PFRV showed only 9.2% (23/250 fry) mortality during 18 days observation (Bootsma *et al.*, 1975). Meier (1985) also reported that VHS virus showed less virulence with increasing age of pike fish.

Routes of entry are also related to virulence of fish viruses. A direct injection challenge generally exhibits more virulence than a bath challenge. Ahne (1978) reported that carp which received an intra-peritoneal injection with 10^3 pfu/fish of SVCV showed 90% mortalities, while carp which received a 2 h bath with 4×10^4 pfu/ml had 20% mortalities. These experiments were conducted at 13°C for 30 days. Mortalities of VHSV-injected sea bass and turbot were also found to be higher than those of VHSV-bathed fishes (Castric and de Kinkelin, 1984). However, the waterborne infection route would represent the most likely route of viral entry, especially in fish hatcheries and culture ponds. Yamamoto *et al.* (1992) conducted an *in vitro* infection of rainbow trout gills, fins, and abdominal skin with VHSV and IHNV. Both viruses had the ability to infect and multiply in these tissues and the authors suggested that the viruses could easily enter the fish body by way of these epidermal tissues.

The degree of virulence of viruses can be expressed as 50% lethal dose (LD_{50}) which is the amount of virus causing 50% mortality in experimental fish. Engelking and Leong (1989) performed experimental bath challenges with IHNV against 2 fish species for 30 days at 12°C. The LD_{50} of 5 isolates of IHNV against rainbow trout fry were found to be between 2.62 $\log_{10}$ and 3.70 $\log_{10}$ $TCID_{50}$/ml. The viruses used in the experiment were isolated from different fish farms in North America and had different protein

profiles in gel electrophoresis. It was also reported that the LD_{50} of 2 isolates of IHNV against sockeye salmon fry were 3.26 $\log_{10}$ and 3.32 $\log_{10}$ $TCID_{50}$/ml.

Fish viruses have a limited ability to cause disease or death in wild or farmed fishes. The amount of virus in surviving fish usually either decreases or disappears after some period following infection. Bootland *et al.* (1994) found a decrease in virus isolation from IHNV-infected brook trout fry at 12°C. Fish fry which received a high dose (10^6 pfu/ml) bath challenge of IHNV isolate RB showed a 35% cumulative mortality. The surviving fry with normal appearance were taken for virus isolation. The authors reported that the percent of virus isolation at weeks 1, 3, 5 and 8 were 89%, 8%, 0% and 0%, respectively. Fish fry given a low dose (10^2 pfu/ml) bath challenge showed no mortality and no virus could be isolated. A decrease in virus was also reported in SVCV-infected carp (Ahne, 1986). Carp weighting 750 g were bathed with SVCV at 10^3 pfu/ml for 2 h at 10°C. These fish were separated into 3 groups and held at 10, 15 and 20°C. Virus was detected in the blood of infected carp for at least 12 weeks post-infection at 10°C while virus in the other 2 groups disappeared after 1 week at 15°C and 20°C holding temperatures. Frantsi and Savan (1971) conducted virus isolation from surviving brook trout fry 64 days post-infection with IPNV. The virus could be isolated from ~25% of the surviving fish.

From the field investigations of this present study, rhabdoviruses were isolated only from diseased fishes during the early period of the outbreak. The rhabdovirus may be one of the etiological agents that initiates an onset of EUS and the role of rhabdovirus needs to

be established. It is important to evaluate the persistence and virulence of rhabdovirus in experimentally infected fishes at different ages and temperatures.

4.2 Objectives:

- To study the persistence of rhabdovirus in juvenile striped snakehead held at 20±0.5°C.
- To compare the virulence of rhabdovirus in juvenile striped snakehead held at 20±0.5°C and 29±2°C.
- To compare the virulence of rhabdovirus in striped snakehead fry held at 20±0.5°C and 28±1°C.
- To compare the virulence of rhabdovirus in 4 fish species held at 20±0.5°C and 28±1°C.
- To determine the LD_{50} of rhabdovirus to striped snakehead fry held at 20±0.5°C

4.3 Materials and Methods

Virus and Virus Titration

Rhabdovirus stain T9412 was isolated from muscle tissue beneath an ulcerative lesion of an EUS-affected juvenile striped snakehead fish collected in January 1994 from a rice paddy field located in Bangkok (Chapter 3). Virus stocks were prepared in HCK and BF-2 cells and stored at 4°C. These preparations were serially diluted in 10-fold steps from 10^{-1} to 10^{-12} in sterile vials by first dispensing 900 µl of L-15 medium with 2% FCS

and 1× antibiotics into a series of vials and adding 100 µl to the first vial (10^{-1}). The virus was mixed well and serial dilutions then prepared across the rest of the vials.

A confluent monolayer each of HCK and BF-2 cells in 25 cm^2 flasks was trypsinised and the cells resuspended in 10 ml of L-15 medium (2% FCS, 1× antibiotics). Cells were seeded into 96-well plates using an 8-channel autopipette. Each well received 100 µl of single cell suspension. On the plastic lid, column numbers 1 to 12 were labeled as 10^{-1} to 10^{-12} virus dilution, respectively. Simultaneous inoculation of cells with virus was performed by inoculating 100 µl of each virus dilution into 6 replicate wells (rows A-F) per column. Wells in rows G and H were inoculated with 100 µl of L-15 medium (2% FCS, 1× antibiotics) and labelled as controls. Plates were covered and sealed using flexible film and incubated at 23°C. Cultures were observed daily under an inverted microscope for CPE and $TCID_{50}$ calculated by the Spearman-Kärber method (Kärber, 1931) (Appendix 1).

The first stock of rhabdovirus isolate T9412 used for experimental infection was obtained from third passage in BF-2 cells with 9.0 $\log_{10}$ $TCID_{50}$/ml. The other stock was obtained from fourth passage in HCK cells with 7.83 $\log_{10}$ $TCID_{50}$/ml.

Experimental Fishes

Striped snakehead juveniles (*Channa striatus* Bloch)**:** Two and a half month old snakehead fish averaging 24.45 ± 3.72 g (mean ± standard error, n = 10) were obtained from a nursery fish farm in Amphur Song-pee-nong, Suphanburi province in early December 1993. This farm had severe losses due to EUS in 1991-1992, but no outbreak

was recorded in 1992-1993 or 1993-1994. The fish were transported to AAHRI, Bangkok and maintained in a wet laboratory at room temperature (28±1°C). The fish were held in glass aquaria containing 150 litres dechlorinated tap water and treated immediately with 1% salt for 2 days. On day 3, 50% of the water was changed and the concentration of salt maintained at ~0.5% for the next 7-10 days. During this period, the water was changed every 1 or 2 days and the fish were treated once with 45 ppm formalin. Fish were fed with commercial feed containing 30% protein (snakehead fish pellet feed, Chareon Pokaphan Ltd, Thailand). Fish were acclimated for more than 2 months and very healthy before experimental use.

Striped snakehead fry: Two batches of snakehead fish fry averaging ~2.0 cm (7 fry/g) and ~2.5 cm (6 fry/g) were caught from a small pond behind AAHRI on 21st March 1994 and 27th April 1994.

Silver barb fry (*Puntius gonionotus*): Obtained from the hatchery of the National Inland Fisheries Institute (NIFI), Bangkok and were ~1.5 cm in total length (12 fry/g).

Hybrid catfish fry (*Clarias gariepinus* × *C. macrocephalus*): Obtained from a private hatchery in Bangkok and were ~2.5 cm in total length (4 fry/g).

Young male guppy fish (*Poecilia reticulata*): Obtained from a private pet fish hatchery in Nakon-prathom province, central Thailand, and were ~2.0 cm in total length (8 fry/g).

There were no reports of EUS in the AAHRI pond or any of the hatcheries. All fishes were brought to the wet laboratory 1 day before experiments started and were not fed during the experimental period.

4.3.1 Persistence of Rhabdovirus in Juvenile Striped Snakehead Held at 20±0.5°C

Ninety striped snakehead juveniles were randomly selected and transferred to acclimate in 4 glass aquaria within a temperature-controlled room at 20±0.5°C. Each aquarium contained 100 litres water and held 20 fish. A static water system was applied. Two days acclimatisation was allowed before the challenge experiments started. No water aeration was given throughout the experiment.

Challenges were performed using intraperitoneal (i/p) and intra-muscular (i/m) injections and bath exposure to rhabdovirus isolate T9412 as follows:

- Aquarium A: Twenty fish received i/p injection of virus at 8.0 $\log_{10}$ $TCID_{50}$/0.1 ml/fish.
- Aquarium B: Twenty fish received i/m injection of virus at 8.0 $\log_{10}$ $TCID_{50}$/0.1 ml/fish.
- Aquarium C: Thirty fish were bathed in a bucket with 2 litres of water containing 2 ml of virus stock (~6.0 $\log_{10}$ $TCID_{50}$/ml) for 2 h and then transferred to the aquarium.
- Aquarium D: Twenty fish received i/p injection of 0.1 ml L-15 medium (2% FCS, 1× antibiotics) and were maintained as a control group.

Fish were fed once a day. Fifty litres of aquarium water was changed twice a week. The contaminated water was drained into an underground cement tank and treated with 30 ppm chlorine (calcium hypochlorite). After challenge, 4 fish each from Aquaria A, B and C and 2 fish from Aquarium D were removed on days 3, 7, 14, and 30 for virus isolation. Sets of 2 fish from the same aquarium were sacrificed and the muscle tissues located just posterior to the vent of both fish were pooled as 1 sample. A second sample was obtained from pooled tissue of internal organs (kidney, spleen, liver, intestine and pancreas) from the same 2 fish. The weight of pooled tissue sample was ~1 g. Tissue preparation and virus isolation were carried out according to the standard method described previously (Chapter 3).

Tissue extracts (1:50 dilution) were simultaneously inoculated into 24-well plates of HCK and BF-2 cell lines. Each sample was inoculated into 2, 3 or 4 replicate wells as appropriate. The same number of replicate wells in every plate were inoculated with L-15 medium (2% FCS, 1× antibiotics) as controls. The FHM line was also used to isolate the virus from virus-bathed fish sampled on day 14. Cell cultures were observed daily for at least 2 weeks. The first passage of culture fluids was carried out on day 10. Successful virus isolation was determined by cytopathic effect changes in the cell lines at first passage. Percentage virus isolations were recorded and the persistence of virus following different routes of infection were compared.

4.3.2 Virulence of Rhabdovirus in Juvenile Striped Snakehead Held at 20±0.5°C and 29±2°C

Seventy striped snakehead juveniles were transferred to 3 glass aquaria containing 100 litres of clean water in the temperature-controlled room at 20±0.5°C. They were allowed a further 2 days acclimatisation. The fish were inoculated as follows:

- Aquarium A: Thirty fish received i/p injection of virus at 6.83 $\log_{10}$ $TCID_{50}$/0.1 ml/fish and were held throughout in the temperature-controlled room.
- Aquarium B: Twenty fish received i/p injection of virus at 6.83 $\log_{10}$ $TCID_{50}$/0.1 ml/fish and were held in the temperature-controlled room for 6 days, then moved to a fibreglass aquarium containing 100 litres of water located behind the AAHRI building (29±2°C).
- Aquarium C: Twenty fish received i/p injection of L-15 medium (2% FCS, 1× antibiotics) at 0.1 ml/fish and were held in the temperature-controlled room as a control group.

The fish in all aquaria were fed daily. Fifty percent of water was changed twice a week. A static water system without aeration was used. Fish behaviour, clinical changes and mortalities were observed daily for 30 days.

4.3.3 Virulence of Rhabdovirus in Striped Snakehead Fry Held at 20±0.5°C and 28±1°C

Eighty striped snakehead fry averaging 2.5 cm total length were used. The fish were held in 4 small aquaria each containing 20 fish. Two aquaria were held in the temperature-controlled room at 20±0.5°C and the other 2 held in the wet laboratory at 28±1°C. One aquarium at each holding temperature was subjected to a bath challenge with rhabdovirus and the other 2 remained as control groups. For the rhabdovirus-bathed group, fish were held in 1 litre of water containing 1 ml of 7.83 $\log_{10}$ $TCID_{50}$/ml rhabdovirus isolate T9412. For the control group, fish were held in 1 litre of water containing 1 ml L-15 medium (2% FCS, 1× antibiotics). After 2 h, 4 litres of clean water were added to each aquarium. Fish behaviour and mortalities were observed daily. The virulence of rhabdovirus at different incubation temperatures was compared.

4.3.4 Virulence of Rhabdovirus in 4 Fish Species Held at 20±0.5°C and 28±1°C

Four fish species comparing striped snakehead fry (~2.0 cm), silver barb fry (~1.5 cm), hybrid catfish fry (~2.5 cm) and young male guppy fish (~2.0 cm) were used. Two experiments were set up at the same time, one at 20±0.5°C and one at 28±1°C. For each experiment, 40 fish of each species were randomly selected. Two small aquaria were used for each fish species, each holding 20 fish. One aquarium of each species was subjected to bath challenge with rhabdovirus and the other was assigned as a control group. For the rhabdovirus-bathed group, fish were held in 1 litre of water containing 1 ml of 7.83 $\log_{10}$ $TCID_{50}$/ml rhabdovirus isolate T9412. For the control group, fish were

held in 1 litre of water containing 1 ml L-15 medium (2% FCS, 1× antibiotics). After 2 h, 4 litres of clean water were added to both aquaria of each fish species. Fish behaviour and mortalities were observed daily. Virulence of the rhabdovirus in different fish species at different incubation temperatures was compared.

4.3.5 Determination of LD_{50} of Rhabdovirus for Striped Snakehead Fry Held at 20±0.5°C

One hundred and sixty striped snakehead fry (~2.0 cm) were randomly selected. The experiment was conducted in the temperature-controlled room at 20±0.5°C. Stock rhabdovirus isolate T9412 containing 7.83 $\log_{10}$ $TCID_{50}$/ml, was first diluted 1:20 with L-15 medium containing 2% FCS and 1× antibiotics. A further 6 serial 1:2 dilutions were then prepared giving a total of seven 2-fold dilutions of rhabdovirus from 1:20 to 1:1280. One ml of each virus dilution was added to each of 7 small aquaria containing 1 litre of dechlorinated tap water giving a further 1:1000 dilution. The final dilution of rhabdovirus in the aquaria were therefore 1:20000, 1:40000, 1:80000, 1:160000, 1:320000, 1:640000 and 1:1280000. For the control aquarium, 1 ml of L-15 medium (2% FCS, 1× antibiotics) was added instead of virus.

Fish fry were randomly distributed to the 8 aquaria. Each aquarium held 20 fry. Fry were allowed to incubate for 2 h, then, 4 litre of dechlorinated tap water was added to each aquarium. Fish behaviour and mortalities were observed daily for 11 days. No feed was given throughout the experiment. The amount of rhabdovirus causing 50% death (LD_{50}) in snakehead fish fry was calculated as shown in Appendix 2.

4. 4 Results

4.4.1 Persistence of Rhabdovirus in Juvenile Striped Snakehead Held at 20±0.5°C

During the 30-day observation period, some clinical changes occurred in the experimental groups of fish. The most noticeable changes were observed in fish which had received i/m injection. These fish showed darkening of the skin at the site of injection and most developed small lesions within the first week. Some fish developed shallow haemorrhagic ulcers with swollen edges ~1.5 cm in diameter with an opening of 0.5 cm. The hypodermal tissue of affected fish was exposed to the water. Moribund fish were found to have developed extensive haemorrhage in skeletal muscle, internal organs and abdominal cavity. A few fish with shallow ulcers showed an ability to heal. Healing ulcers were dark in colour, less swollen and decreased in size.

Only minor changes were found in fish with i/p injection and bath challenge.

Percentage of successful virus re-isolation was found to decrease over each successive period of sampling as summarised in Table 4.1. Details of fish samples and virus isolation are follows:

Sampling on day 3:

- Four fish sampled from the i/p injected group appeared normal except for lethargy and reduced active movement. Internal organs and skeletal muscle were normal. Virus was isolated from 100% of samples.

Table 4.1 Observation of CPE and percent virus isolation from pooled tissue from pairs of striped snakehead fish juveniles challenged with rhabdovirus T9412 at 20±0.5°C. The supernatants (1:50 dilution) of tissue extract were simultaneously inoculated into cell cultures. Controls (not shown) were inoculated with L-15 medium.

Days & Cell line	Culture passage	Virus isolation from snakeheads challenged with rhabdovirus T9412											
		i/p with 8.0 $\log_{10}$ $TCID_{50}$ /0.1 ml/fish				i/m with 8.0 $\log_{10}$ $TCID_{50}$/0.1 ml/fish				bath with 6.0 $\log_{10}$ $TCID_{50}$ /ml for 2 h			
		1	2	3	4	5	6	7	8	9	10	11	12
		mus	int	mus	int	mus	int	mus	int	mus + gill	int	mus + gill	int
Day3		100% virus isolation				100% virus isolation							
HCK	ini	+	+	–	+	+	+	+	?				
	1 st	+	+	–	+	+	+	+	+			nd	
BF2	ini	+	+	+	+	+	+	+	+				
	1 st	+	+	+	+	+	+	+	+				
Day7		75% virus isolation				50% virus isolation				50% virus isolation			
HCK	ini	–	?	+	+	–	?	–	?	?	?	+	?
	1 st	+	–	+	+	+	+	–	–	–	–	+	–
BF2	ini	–	–	+	+	+	–	?	–	?	+	+	?
	1 st	–	–	+	+	+	+	–	–	–	+	+	–
Day14		25% virus isolation				50% virus isolation				0% virus isolation			
HCK	ini	–	+	–	+	+	?	–	?	?	?	?	?
	1 st	–	–	–	–	+	–	–	–	–	–	–	–
BF2	ini	–	?	–	?	+	+	?	?	–	–	–	–
	1 st	–	–	+	–	+	+	–	–	–	–	–	–
FHM	ini	nd	nd	nd	nd	nd	nd	nd	nd	–	–	–	–
	1 st	nd	nd	nd	nd	nd	nd	nd	nd	–	–	–	–
Day30		0% virus isolation				25% virus isolation				0% virus isolation			
HCK	ini	–	?	–	?	+	–	–	?	–	–	–	–
	1 st	–	–	–	–	+	–	–	–	–	–	–	–
BF2	ini	–	?	–	–	+	–	–	?	?	–	–	–
	1 st	–	–	–	–	+	–	–	–	–	–	–	–

Control fish were sampled together with the infected fish and no virus was isolated.
ini = initial inoculation; mus = muscle tissue sample; int = internal organ tissue sample;
1 st = first passage; – = negative CPE; ? = doubtful CPE; + = positive CPE; nd = not done

- Four fish sampled from the i/m injected group had developed haemorrhagic lesions at the site of injection. Extensive haemorrhages were found in skeletal muscle, internal organs and abdominal cavity. Virus was isolated from 100% of samples.
- The bath-challenged group was not sampled at this time but a few fish showed discoloration of the skin, grey to white patches on the body and ragged fins.

Sampling on day 7:

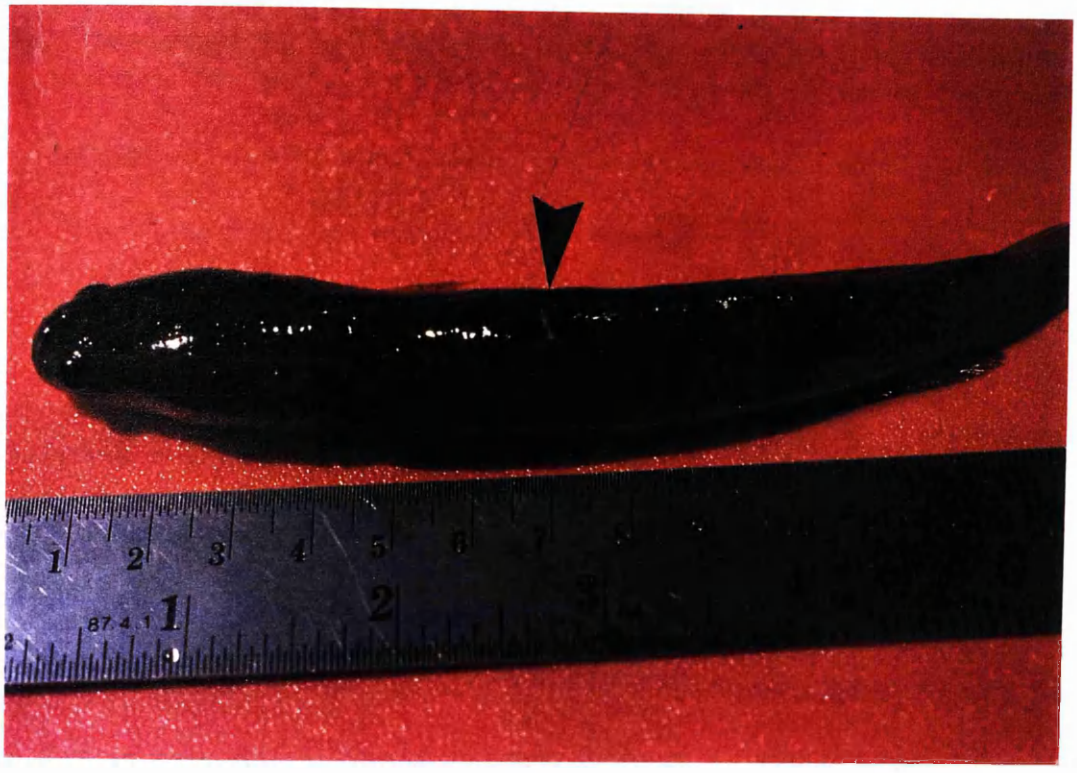
- Four fish sampled from the i/p injected group had a normal appearance. Internal organs and skeletal muscle were both normal. Virus was re-isolated from 75% of samples.
- Four fish were taken from the i/m injected group. Two fish had shallow haemorrhagic ulcers (Figure 4.1) with severe haemorrhage inside the body. The other 2 had no external lesions but focal haemorrhages were found in the muscle beneath the injection site. Virus was re-isolated from 50% of samples.
- Four fish were taken from the bath-challenged group. One specimen had discoloration of the skin. All fish had normal internal organs and skeletal muscle. Virus was re-isolated from 50% of samples.

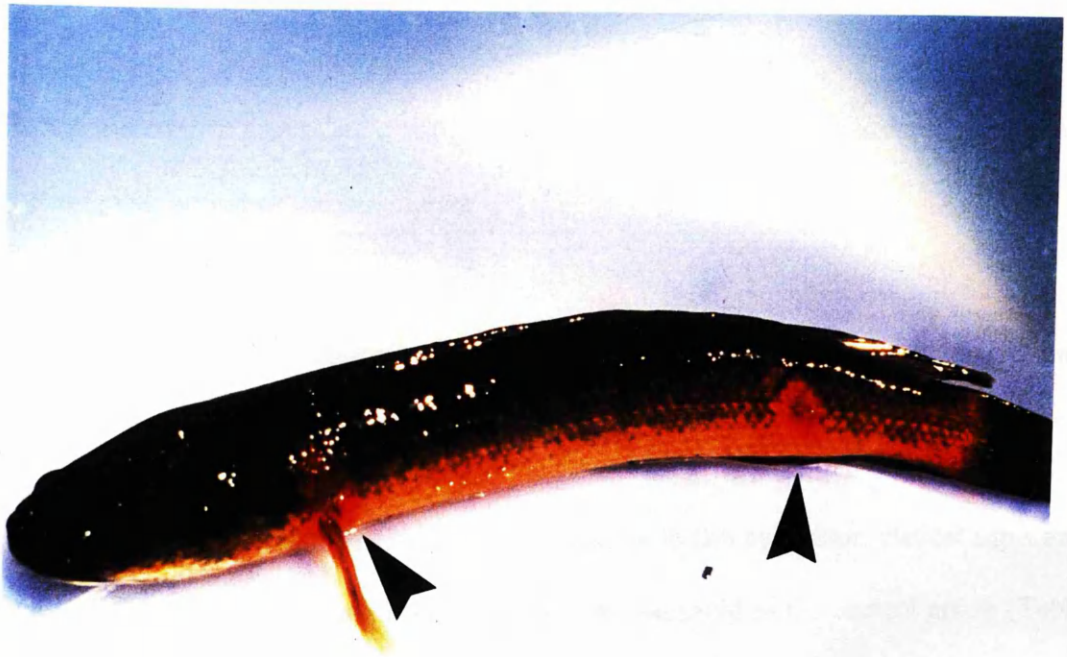
Sampling on day 14:

- Four fish were taken from the i/p injected group. One fish had developed a few red haemorrhagic patches on the body (Figure 4.2) but internal organs appeared normal. Virus was re-isolated from 25% of samples.

Figure 4.1 Striped snakehead fish receiving i/m injection of rhabdovirus showing haemorrhagic shallow ulcer (arrow) at the site of injection on day 7 post-infection at 20±0.5°C.

Figure 4.2 Striped snakehead fish receiving i/p injection of rhabdovirus showing few focal haemorrhages (arrows) on day 14 post-infection at 20±0.5°C.





- Four fish were taken from the i/m injected group. One fish had a small open lesion. The other 3 had no external lesions but were found to have minor haemorrhage underneath the skin at the site of injection. Virus was re-isolated from 50% of samples.
- Four fish taken from the bath-challenged group had a normal appearance. No virus was isolated from these fish.

Sampling on day 30:

- Fish sampled from the i/p injected and bath-challenged groups appeared normal. No virus was isolated from these groups.
- Two fish in the i/m injected group still had small lesions at the site of injection, while the rest were normal. The wounds were smaller in size with less haemorrhage and less severe than at earlier sampling times (Figure 4.3). Virus was re-isolated from 25% of samples.

Fish in the control group had a normal appearance and no virus was isolated throughout the sampling period of the experiment.

4.4.2 Virulence of Rhabdovirus in Juvenile Striped Snakehead at 20±0.5°C and 29±2°C

During the 30-day observation period, some changes in fish behaviour, clinical signs and mortalities were observed in the virus-injected fish compared to the control group (Table 4.2). Fish in aquarium A which had received i/p injection and were held at

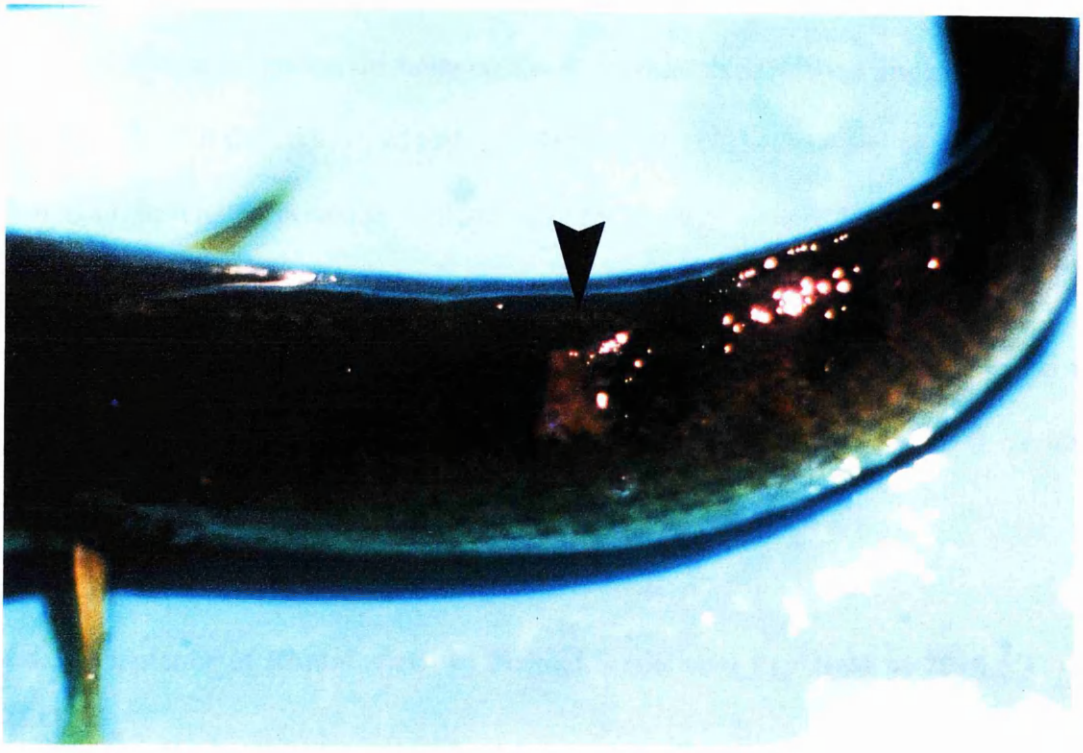
Table 4.2 Behaviour, clinical signs and cumulative mortalities of striped snakehead juveniles (24.45 g ± 3.72 SE) injected with rhabdovirus at 6.83 $\log_{10}$ $TCID_{50}$/0.1 ml/fish. Fish were held at 20±0.5°C and 29±2°C. Control group received i/p injection with L-15 (2% FCS and 1× antibiotics) and was held at 20±0.5°C.

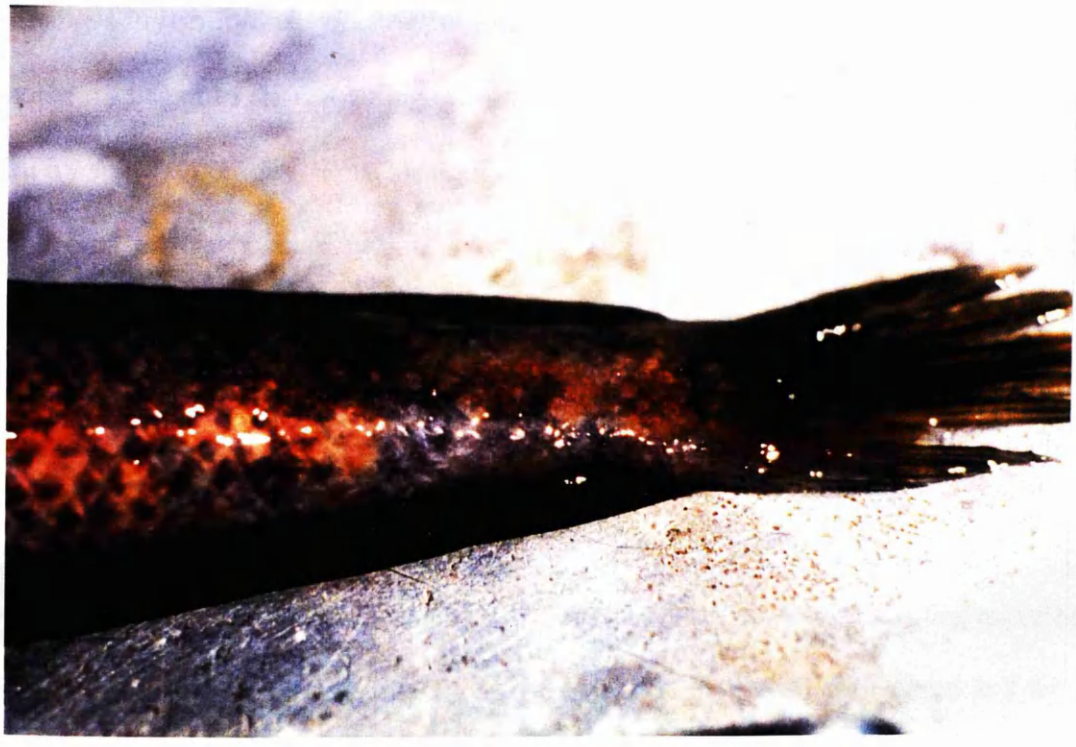
Days	Aquarium A: i/p injection with rhabdovirus and held at 20±0.5°C	Aquarium B: i/p injection with rhabdovirus and held 20±0.5°C then 29±2°C	Aquarium C i/p injection with L-15 medium and held at 20±0.5°C
Days 1-2	Slow movement.	Slow movement.	Normal.
Day 5	Some fish with ragged fins.	Some fish with ragged fins.	Normal and fed well.
Day 6	1 fish died with petechia haemorrhage and ragged fins	**Transferred to a new aquarium at 29±2°C.**	Normal and fed well.
Day 7	Start to feed.	Hyperactive movement.	Normal and fed well.
Day 9	Normal.	1 fish died with petechial haemorrhage.	Normal and fed well.
Day 12	1 fish died with swollen and haemorrhagic eyes.	Some fish with discoloured skin.	Normal and fed well.
Day 15	Normal and fed well.	Fish fed well.	Normal and fed well.
Day 17	Normal and fed well.	Normal and fed well.	Normal and fed well.
Day 22	Normal and fed well.	5 fish with petechial haemorrhage.	Normal and fed well.
Day 26	Normal and fed well.	Fish returned to normal.	Normal and fed well.
Day 30	28 fish normal and fed well.	19 fish normal and fed well.	Normal and fed well.
	6.7% (2/30) cumulative mortality.	5.0% (1/20) cumulative mortality.	0% mortality.

20±0.5°C throughout showed slower movement during the first few days post-infection. Infected fish also developed ragged fin rays on day 5. Most fish returned to normal after day 9. Only 2/30 fish were found dead during the observation period. One fish died on day 6 showing ragged fins and petechial haemorrhages on the body (Figure 4.4). The second fish was found on day 12 showing exopthalmia and haemorrhage of the eyes. Cumulative mortalities were 6.7%. Fish in aquarium B received the same virus injection but were only held at 20±0.5°C for 6 days. During these first 6 days, the

Figure 4.3 Striped snakehead fish receiving i/m injection of rhabdovirus showing small wound with less haemorrhage and no swelling (arrow) at the site of injection on day 30 post-infection at 20±0.5°C.

Figure 4.4 Striped snakehead fish receiving i/p injection of rhabdovirus showing ragged fin and petechial haemorrhages on body surface on day 6 post-infection at 20±0.5°C.





fish showed the same behaviour and clinical signs as those in aquarium A. On transfer to 29±2°C, the fish showed hyperactive movement for a day. Only 1 fish died with petechial haemorrhages on the body on day 9. Some fish developed discoloured skin on day 12. The fish then recovered and returned to normal behaviour and appearance. Five fish again developed petechial haemorrhages on the body on day 22 but no further skin damage or mortality was observed. These 5 fish recovered and returned to normal before the end of the experiment. Cumulative mortalities were 5.0%. Both infected fish groups had approximately the same percent cumulative mortalities. Controls remained normal and no mortality was observed throughout the experiment.

4.4.3 Virulence of Rhabdovirus in Striped Snakehead Fry Held at 20±0.5°C and 28±1°C

Rhabdovirus-bathed striped snakehead fry showed changes in behaviour and mortalities. Changes in fish fry behaviour were first observed 3 days post-infection. At 20±0.5°C, some fry began to show swirling movement and 1 fish died on day 4. A few days later all fry showed non-directional swirling movement. A few fry swam ventral side upwards and died in this position near the water surface. The number of mortalities rapidly increased on days 6-9 (Table 4.3). Most of the dead fry exhibited curved bodies and lay on the bottom of the aquarium. A 100% mortality was observed by day 9 in the virus-bathed group held at 20±0.5°C.

As with the 20°C group, virus-bathed fish fry held at 28±1°C showed swirling movement but fewer fry were affected and with less severity. Mortalities first occurred in 3 fry on

day 6. By the end of the experiment on day 10, cumulative mortality was 5/20 (25%). The fish fry of both control groups held at 20±0.5°C and 28±1°C appeared normal and no mortality was observed throughout 10 days experiment.

Table 4.3 Cumulative mortalities of striped snakehead fry bathed with ~4.83 log_{10} $TCID_{50}$/ml of rhabdovirus* isolate T9412 and held at 20±0.5°C and 28±1°C for 10 days. Controls for both incubation temperatures were bathed with L-15 medium (2% FCS, 1× antibiotics).

Group	No. of fry	Cumulative mortality of rhabdovirus-bathed striped snakehead fry						
		day 3	day 4	day 6	day 7	day 8	day 9	day 10
20±0.5°C								
Virus bath	20	–	1	6	11	15	20	20
control	20	–	–	–	–	–	–	–
28±1°C								
Virus bath	20	–	–	3	3	4	4	5
control	20	–	–	–	–	–	–	–

* The stock rhabdovirus was propagated in HCK cell line and had 7.83 log_{10} $TCID_{50}$/ml. Fish were bathed with 1 litre of water containing 1 ml of virus stock.

4.4.4 Virulence of Rhabdovirus in 4 Fish Species Held at 20±0.5°C and 28±1°C

The virulence study with snakehead fry showed that the rhabdovirus was more virulent at the lower temperature of 20±0.5°C (section 4.4.3). The experiment was repeated with a second batch of striped snakehead fry averaging 2.0 cm in length, silver barb fry, hybrid catfish fry and young male guppy fish. During 10 days observation, only the

striped snakehead fry held at 20±0.5°C exhibited clinical signs and mortalities with a similar degree of severity as observed previously with striped snakehead fry held at 20±0.5°C. A 100% mortality was observed by day 8 (Table 4.4). A few virus-bathed striped snakehead fry held at 28±1°C showed some changes in behaviour. Whirling movement was observed on day 5 but affected fry returned to normal and no mortality occurred. The rhabdovirus-bathed silver barb fry, hybrid catfish fry and young male guppy fishes held at both temperatures showed no change in behaviour or mortality compared to the control fish through 10 days observation.

Table 4.4 Cumulative mortalities of striped snakehead fry, silver barb fry, hybrid catfish fry and young male guppy fish bathed with ~4.83 $\log_{10}$ $TCID_{50}$/ml of rhabdovirus* isolate T9412 and held at 20±0.5°C and 28±1°C for 10 days. Controls of all species were bathed with L-15 medium (2% FCS, 1× antibiotics) and kept at 2 incubation temperatures. Controls remained normal with no mortality (not shown).

Fish species	Incubation temp.	No. of fish	Cumulative mortalities of rhabdovirus-bathed fishes					
			day 1	day 3	day 5	day 6	day 8	day 10
Striped snakehead	20°C	20	–	2	15	17	20	20
	28°C	20	–	–	–	–	–	–
Silver barb	20°C	20	–	–	–	–	–	–
	28°C	20	–	–	–	–	–	–
Hybrid catfish	20°C	20	–	–	–	–	–	–
	28°C	20	–	–	–	–	–	–
Guppy fish	20°C	20	–	–	–	–	–	–
	28°C	20	–	–	–	–	–	–

* The stock rhabdovirus was propagated in HCK cell line and had 7.83 $\log_{10}$ $TCID_{50}$/ml. Fish were bathed with 1 litre of water containing 1 ml of virus stock.

4.4.5 Determination of LD_{50} of Rhabdovirus to Striped Snakehead Fry Held at 20±0.5°C

Striped snakehead fish fry bathed with rhabdovirus dilutions from 1:20000 down to 1:320000 showed a similar response and mortalities. Whirling movement was a major response to the rhabdovirus and was first observed on days 2-3. Many fry died the following day. Mortalities then rapidly increased and reached 100% on days 7-11 (Table 4.5). One fish bathed with rhabdovirus dilution at 1:640000 also showed whirling movement and was found dead on day 3 while the remainder were unaffected through 11 days observation. Fry bathed with rhabdovirus dilution 1:1280000 were also unaffected. Both unaffected and control groups showed normal behaviour. The amount of rhabdovirus and mortalities of fish fry were used to calculate LD_{50} according to Kärber (1931) as shown in Appendix 2. The LD_{50} within 11 days at 20±0.5°C of rhabdovirus isolate T9412 against striped snakehead fish fry averaging 2.0 cm was 2.16 $\log_{10}$ $TCID_{50}$/ml.

Table 4.5 Cumulative mortalities of striped snakehead fry (~2.0 cm in length) bathed with different dilutions of rhabdovirus* isolate T9412 and held at 20±0.5°C. The LD_{50} within 11 days was 2.16 $\log_{10}$ $TCID_{50}$/ml as calculated by the method of Spearman -Kärber (Kärber, 1930).

Virus dilution	No. of fry	Cumulative mortality of rhabdovirus-bathed striped snakehead fry							
		day 1	day 3	day 4	day 6	day 7	day 9	day 10	day 11
1:20000	20	–	4	10	19	20	20	20	20
1:40000	20	–	3	8	15	17	18	19	20
1:80000	20	–	1	4	4	11	19	20	20
1:160000	20	–	–	9	9	17	19	19	20
1:320000	20	–	–	7	14	15	20	20	20
1:640000	20	–	1	1	1	1	1	1	1
1:1280000	20	–	–	–	–	–	–	–	–
Control	20	–	–	–	–	–	–	–	–

* The stock rhabdovirus was propagated in HCK cell line and had 7.83 $\log_{10}$ $TCID_{50}$/ml.

4. 5 Discussion

The persistence of rhabdovirus isolate T9412 in experimentally infected juvenile snakehead fish decreased over the period of sampling. Virus was isolated from all tissue extract samples from both i/p- and i/m-injected fish on day 3 post-infection. Percentage of virus isolation from fish infected by the different routes then decreased to 50-75% on day 7, 0-50% on day 14 and 0-25% on day 30. These results suggest that virus may be partially or entirely eliminated by the host defense system and the surviving fish may develop antidody response to the virus as some evidence from surviving fish from other rhabdovirus infections has shown the development of a humoral immune response

(Ristow *et al.*, 1993; Lorenzen *et al.*, 1993). Similar decreases in virus isolation were recorded in IHNV-infected brook trout fry by Bootland *et al.* (1994) in which virus recovery decreased to 89% on day 7, 8% on day 21 and 0% on day 35. The persistence of VHS virus in rainbow trout was found to decrease with increasing temperature from 5°C to 20°C (Jørgensen, 1982b). Although the amount of rhabdovirus T9412 used in the present virus persistence study was very high of 8.0 $\log_{10}$ $TCID_{50}$/0.1 ml/fish for injection and 6.0 $\log_{10}$ $TCID_{50}$/ml for bathing, no mortality occurred during the course of sampling.

The decrease in virus isolation from experimentally infected fish closely followed the pattern of isolation from naturally infected fish. In the study reported in Chapter 3, virus was only isolated during the first 2 weeks of the EUS outbreak. For the experimentally infected fish at a constant temperature at 20±0.5°C, virus recovery at 2 weeks post-infection had fallen to 0% for bath challenge, 25% for i/p challenge and 50% for i/m challenge groups. At week 4, no virus was isolated from i/p or bath challenged groups. Virus was isolated from only 1 of 4 i/m challenge samples which contained a piece of muscle tissue at the ulcerated lesion site of the fish (Figure 4.3). The rapid decrease of virus in the fish host immediately suggests how difficult it might be to isolate virus from naturally EUS-diseased fishes as previously encountered by Frerichs *et al.* (1989b) and Roberts *et al.* (1994).

Rhabdovirus isolate T9412 generally caused only minor clinical changes in snakehead fish juveniles and only few fish developed a severe response. The changes depended on the routes of challenge. Bath-challenged fish developed grey to white patches on the

body, discoloration of the skin and damaged fin rays. I/P-injected fish became lethargic and developed ragged fins, discoloration of the skin, petechial haemorrhages on the body, focal haemorrhages on the peduncle and pectoral areas and exopthalmia. I/M-injected fish became lethargic and developed shallow haemorrhagic ulcers and haemorrhages in the skeletal muscle, internal organs and abdominal cavity. Entering via the i/m route, isolate T9412 was found to cause the most severe clinical signs in the juvenile stage of striped snakehead fish. Entering via i/p and bath routes, the virus caused changes in the juvenile fish skins which showed some degree of similarity to the pathological findings described by Wattanavijarn *et al.* (1985), Saitanu *et al.* (1986) and Frerichs *et al.* (1993). Interestingly, the studies of these 3 groups of workers and the present study used different EUS-associated viruses and test conditions. The partial similarity of clinical signs suggest that the EUS-associated viruses cause at least some damage to the outer protection (skin) of infected fishes.

The EUS-isolated rhabdovirus T9412 caused very low mortality in striped snakehead fish juveniles. Cumulative mortalities after 30 days for fish injected intra-peritoneally with 6.83 $\log_{10}$ $TCID_{50}$/0.1 ml/fish were 6.7% at 20±0.5°C and 5% at 29±2°C. An increase in water temperature from 20°C to 29°C did not seem to decrease virulence for fish juveniles but it did so for fish fry. A very low virulence of rhabdovirus with no mortality was also recorded by Frerichs *et al.* (1993) who found little skin damage in few snakehead fish juveniles injected with an earlier isolate of rhabdovirus at a dose of 7.3 $\log_{10}$ $TCID_{50}$/0.1 ml/fish or bathed with 5-6 $\log_{10}$ $TCID_{50}$/ml at 20°-25°C.

Surprisingly, isolate T9412 was highly virulent for the fry stage of snakehead fish. An exposure to ~4.83 $\log_{10}$ $TCID_{50}$/ml rhabdovirus started to cause death by day 3 leading to 100% mortality by day 9 at 20°C. Moribund fry showed swirling motion. The same dose of virus causing clinical changes and death in striped snakehead fish fry did not cause any harm or death in silver barb fry, hybrid catfish fry or young guppy fish. The refractory response of the latter 2 species was anticipated as these have never been reported to be affected by EUS in Thailand. In contrast, the refractory response of silver barb fry was unexpected as this species has been known to be severely affected by EUS as well as snakehead fish (Tonguthai, 1985). Silver barb fry may not be an appropriate target species for rhabdovirus isolate T9412 and how they might respond to other EUS-associated rhabdovirus, birnavirus or reovirus isolates still needs to be identified.

EUS-associated rhabdoviurus T9412 has been verified to be more virulent at a low water temperature (20°C) than a high temperature (28°C) which is paralleled by many pathogenic fish viruses such as VHSV (Castric and de Kinkelin, 1984), IPNV (Frantsi and Savan, 1971), IHNV (Amend, 1970) and SVCV (Ahne, 1980). At the preferential temperature of 20°C and with a susceptible host such as striped snakehead fish, the virus caused 100% death in fry but ~5% death in juvenile fish. A greater susceptibility of younger fish to virus infection has also been reported in IPNV-infected rainbow trout (Frantsi and Savan, 1971), PFRV-infected pike (Bootsma *et al.*, 1975) and VHSV-infected pike (Meier, 1985).

The LD_{50} within 11 days at 20°C of rhabdovirus T9412 in striped snakehead fish fry (~2 cm in length or ~0.14 g) was 2.16 $\log_{10}$ $TCID_{50}$/ml. Expressed differently, 1 ml of virus

stock (7.83 log10 $TCID_{50}$/ml) in ~468 litres of water caused 50% mortalities in a snakehead fish fry population within 11 days at 20°C. The LD_{50} from this study was surprising low and may indicate that snakehead fish fry are very sensitive to rhabdovirus infection and that isolate T9412 is pathogenic for fish. From the evidence of this study, the virulence of a rhabdovirus pathogen is dependent on fish species, fish age and temperature. The virulence level of the isolate T9412 seemed high when compared to the LD_{50} values between ~2.62 $\log_{10}$ and ~3.70 $\log_{10}$ $TCID_{50}$/ml of different isolates of IHNV against rainbow trout fry and sockeye salmon fry at 12°C over a 4 weeks experiment (Engelking and Leong, 1989).

The findings of these experimental infections indicate that rhabdovirus alone does not induce typical ulcerative lesions in snakehead fish as seen in naturally EUS-diseased fish. However, the common pathological signs in juvenile fish infected with EUS-associated rhabdovirus were skin and fin damage that may be significant primary lesions opening the door to the attachment and invasion of *Aphanomyces* fungus, as the fungus alone could not invade healthy experimental snakehead fish (Chinabut *et al.*, 1995). These assumptions have been examined in the following chapter of this study.

Chapter 5

Experimental Induction and Transmission of Epizootic Ulcerative Syndrome

5.1 Introduction and Literature Review

Inducing EUS in the laboratory has been very difficult because the 3 interactive disease factors of susceptible fish, appropriate environment and causative agent(s) all need to be present in order to produce the clinical condition (Snieszko, 1974). The fish host factor is uncomplicated as it is recognised that the striped snakehead fish is one of the species most susceptible to natural EUS and is also susceptible to infection with EUS-associated rhabdovirus (Chapter 4), birnavirus (Saitanu *et al.*, 1986) and pathogenic *Aphanomyces* (Roberts *et al.*, 1993) in the laboratory. As epizootics usually occur after heavy rain following the cold period of the year, there is a need to consider factors of low temperature and a dilution effect on alkalinity and hardness of the natural or pond water environment. The causative agent(s) is the most problematic factor that scientists have been investigating since the early years of the epizootics.

Selection of the right infectious agents has been difficult as many infectious agents have been identified from natural epizootics. After the first isolation of *Aphanomyces* fungus from EUS-diseased fish, proof of the organism as a fish pathogen and demonstration of the presence of the fungus in all fish samples with classic EUS (Roberts *et al.*, 1993), the *Aphanomyces* pathogen has been generally accepted as one of the most important

agents, and a declaration of EUS needs to show a presence of *Aphanomyces* infection with necrotic ulceration usually followed by mycotic granuloma (ODA, 1994). However, waterborne challenge of normal snakehead fish with this fungal pathogen did not cause any infection (Chinabut *et al.*, 1995; Roberts, 1994). It is therefore a complex of etiological agents that has been implicated as the causative factor in EUS (ODA, 1994).

From present knowledge, one infectious agent is not enough to induce EUS and additional infectious agent(s) may be needed. It is not surprising that there were no reports of EUS induction in previous infection studies that used a single viral agent in the experimental challenge. Possible EUS induction in the laboratory might have been achieved by infecting snakehead fish with 2 pure culture agents, EUS-associated rhabdovirus and *Aphanomyces* pathogen as first conducted by Millar (1994). However, this study needs to be repeated as most experimental fish were removed during the observation period and only 1 fish was left in the tank developed classic EUS lesions on day 10 (S.D. Millar, pers. comm.).

One of the specific features of EUS is rapid spread of the disease. Once diseased fish appear in natural waters or fish ponds, the condition spreads rapidly to most of the fish population. A few experimental disease transmission studies have been reported that might indicate modes of natural EUS transmission. Balasuriya *et al.* (1990) showed that EUS could be transmitted by co-habitation or feeding with EUS-diseased fish or by holding fish in the water of a naturally affected area in Sri Lanka. The authors found that it took 5-6 days for the feeding method and 7-10 days by co-habitation to induce

signs of EUS. However, the severity of experimentally transmitted disease was less than that in the naturally infected fish and some affected specimens were able to recover. Subasinghe (1993) conducted transmission experiment at 28°C and reported that the introduction of a parasitic protozoa, *Trichodina* sp., could diminish EUS transmission time, but the experimentally infected fish developed only haemorrhages and shallow ulcers similar to the early stage of EUS. However, neither of these 2 reports refers to the presence of fungal hyphae or histopathological observations. A more recent EUS transmission experiment conducted at 23-25°C during the 1991-1992 epizootics in Laguna de Bay, Philippines was reported by Cruz-Lacierda and Shariff (1995). Healthy snakehead fish started to develop disease on days 9-10 after co-habitation with EUS-diseased snakehead fish or after holding in water from Laguna de Bay. A 100% (264/264) infection of EUS-transmitted fish was recorded on day 20 of the experiment. Histopathological examination of transmitted fish revealed mycotic granolomas indicating successful EUS transmission (Cruz-Lacierda and Shariff, 1996).

Previous reports from Saitanu *et al.* (1986) and Frerichs *et al.* (1993) and earlier findings in the present study (Chapter 4) suggest that viruses alone cannot cause the severe ulcerative lesions of EUS but may require the presence of a fungal pathogen. A virus, particularly rhabdovirus, and an *Aphanomyces* pathogen have very specific characteristics that would provide a perfect match for the induction of EUS as follows.

Rhabdovirus was easily isolated from live EUS fishes during the first 2 weeks of the 1994 outbreak (Chapter 3). The pathogenicity of this isolate for snakehead fish was limited to skin damage such as discoloration, petechial and focal haemorrhages and small

ulcers (Chapter 4). Interestingly, the rhabdovirus appeared more virulent at low temperature and could be isolated for only a short period of time from both naturally and experimentally infected fish. Pathogenic *Aphanomyces* was readily isolated from small pieces of muscle tissue beneath the ulcerative lesions using a micro-isolation technique. This agent has the unique characteristics of no growth and death at 37°C and slower growth at 18-31.5°C than saprophytic *Aphanomyces* (Roberts *et al.*, 1993). When a pure culture of this agent was introduced into the muscle of snakehead fish by insertion or injection, it invaded deeply into the fish body and induced mycotic granulomas similar to those in natural EUS-affected fish (Roberts *et al.*, 1993; Chinabut *et al.*, 1995). The most interesting characteristics were that bath challenge with the fungus did not cause any harm to healthy snakehead fish (Chinabut *et al.*, 1995; Roberts, 1994) and that fungus-injected fish showed severe damage of muscle tissue and some mortalities at low temperature (19°C) (Chinabut *et al.*, 1995).

Combining both agents in EUS induction experiments, minor skin damage would be predicted from the rhabdovirus infection and *Aphanomyces invaderis* would take advantage to attach and invade the fish via the damaged skin. Therefore, rhabdovirus isolate T9412 and *Aphanomyces invaderis* strain RF-6 were included as putative etiological agents, striped snakehead fish were chosen as a susceptible fish host and a low incubation temperature such as 20°C represented a reasonable environmental factor. Dechlorinated tap water would also be beneficial for induction and transmission experiments because its freshness, cleanliness and low alkalinity and hardness would represent a few similar water properties after heavy rain. As epizootics usually occur

during the cold period of the year, it was considered of interest to compare the effect of different temperatures on the induction and transmission experiments.

5.2 Objectives:

- To induce EUS in juvenile striped snakehead fish in the laboratory using rhabdovirus isolate T9412 and *Aphanomyces invaderis* type strain RF-6.
- To study EUS transmission in juvenile striped snakehead fish by co-habitation with EUS-induced fish.
- To compare the effect of different temperatures on the induction and transmission experiments

5.3 Materials and Methods

Virus Preparation

Rhabdovirus isolate T9412 was selected for the experiment. The virus had undergone 4 passages in HCK cells post-isolation. Stock virus contained 7.83 $\log_{10}$ $TCID_{50}$/ml. This was the same stock used previously (Chapter 4).

Aphanomyces invaderis Strain RF-6

Aphanomyces invaderis strain RF-6 (Willoughby *et al.*, 1995) was maintained in the AAHRI Mycology Unit. The fungus was originally isolated from EUS-diseased striped snakehead fish collected from Suphanburi province, Thailand, during the 1991-1992 epizootic. Stock fungus mycelium was maintained in glucose-peptone broth medium

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supplemented with 1.0 g/l yeast extract (Roberts *et al.*, 1993) and incubated at 15°C. The mycelium was removed from the stock culture, placed in a 500 ml Erlenmeyer flask containing 200 ml of sterile tap water and incubated on the bench at room temperature. After 4-5 h, spores were produced and released into the water. The spores were separated from mycelium by filtering the water through tissue paper. The spore suspension was counted using a haemocytometer under 400× magnification and diluted to contain 10^8 spores/l with clean dechlorinated tap water. Four litres of spore suspension were prepared for the experiment.

Re-isolation of *Aphanomyces* From Experimental Fish Tissue

The isolation techniques performed were similar to those described by Roberts *et al.* (1993). After sacrifice, the lesion surface on the affected fish was cleaned and dry-wiped using tissue paper and cut away using a 3× flame-sterilised razor blade. A few small pieces of muscle tissue were aseptically removed and incubated in a petri dish containing 30 ml glucose-peptone medium without antibiotics. Incubation was carried out on the bench for 4 h. The pieces of tissue were observed periodically under the light microscope or stereo-microscope for the growth of new hyphal tips. Tissues were dissected and a few tiny pieces of material with viable fungal hyphae were transferred to a plate of glucose-peptone agar containing antibiotics. The plate was sealed using flexible film and incubated at 20±0.5°C for 2-3 days. The fungal hyphae grew, spread out of the tissue and penetrated into the agar. Some bacteria were also present but their spreading was much slower than that of the fungal hyphae. An area containing only fungal mycelium at the edge of the fungal colony was subcultured to a new agar plate to

obtain a pure culture of *Aphanomyces* fungus. Media used were prepared according to Roberts *et al.* (1993).

Experimental Fish

Striped snakehead juveniles averaging 24.45 g (±3.72 SE) were obtained from the same stock as those used previously (Chapter 4).

5.3.1 EUS Induction in Striped Snakehead Fish Juveniles Using Rhabdovirus Isolate T9412 and *Aphanomyces invaderis* Strain RF-6 at 20±0.5°C and 29±2°C

One hundred and sixty striped snakehead juveniles were randomly selected and transferred to acclimate in 8 aquaria within the temperature-controlled room at 20± 0.5°C. Each aquarium contained 100 litres of de-chlorinated tap water and held 20 fish in a static water system. Three days acclimatisation was allowed before the first challenge. No aeration was given throughout the experiments. Stock rhabdovirus at 7.83 $\log_{10}$ $TCID_{50}$/ml was diluted 1:20 with L-15 medium (2% FCS, 1× antibiotics). In the first challenge, each fish received 50 µl i/p and 50 µl i/m of virus or L-15 medium (2% FCS, 1× antibiotics). The total amount of virus in 100µl was ~5.53 $\log_{10}$ $TCID_{50}$/fish. Four aquaria were held at 20±0.5°C in the temperature-controlled room and 4 at 29±2°C outside the building.

The second challenge with *Aphanomyces* spores followed 3 days after the first challenge. The stock spore suspension contained 10^8 spores/l. One litre of this stock was added to each of 4 aquaria containing 100 litres of water to give a final concentration of ~10^6

spores/l or ~10^3 spores/ml. Fish were bathed with spores for 3 days, then 50 litres of the water in the aquaria were changed. All 8 aquaria were coded. The challenge procedures are summarised in Table 5.1.

Table 5.1 Aquaria codes, challenge infectious agents and temperatures used in experimental induction of EUS.

Code	First challenge	Second challenge	Holding temperature	
VS20	inject virus	bath spores	20±0.5°C	
V20	inject virus	–	20±0.5°C	**control**
LS20	inject L-15	bath spores	20±0.5°C	
L20	inject L-15	–	20±0.5°C	**control**
VS29	inject virus	bath spores	29±2°C	
V29	inject virus	–	29±2°C	**control**
LS29	inject L-15	bath spores	29±2°C	
L29	inject L-15	–	29±2°C	**control**

Fish behaviour, clinical signs and mortality were observed. Fish were fed daily and no aeration was given. Fifty percent of the water in the aquaria was changed twice a week. Virus re-isolation was performed as described previously (Chapter 4) except that only internal organs were processed for virus isolation. The filtered tissue extracts (1:50 dilution) were simultaneously inoculated into HCK and BF-2 cell lines. *Aphanomyces* re-isolation was performed as described above.

Successful EUS-induction was determined by the histological identification of granulomas in muscle tissue from beneath the skin lesion caused by *Aphanomyces*. The experiment was terminated on day 30.

5.3.2 EUS Transmission in Striped Snakehead Fish by Co-habitation With EUS-induced Fish at 20±0.5°C and 28±1°C

The experiment was conducted immediately following the EUS induction experiment. Water in aquarium tank VS20 was used in the EUS transmission study. Fifteen striped snakehead juveniles were obtained from the surviving fish in experiment 4.3.1 (Chapter 4) which had been bathed for 2 h with 6.0 $\log_{10}$ $TCID_{50}$/ml of rhabdovirus isolate T9412. Seven weeks after the bath challenge, the fish appeared normal and were transferred to tank VS20 within the temperature-controlled room. On day 12, 8/15 fish developed severe EUS lesions which were confirmed by the presence of fungal granulomas in the muscle tissue in histological sections. These EUS-induced striped snakehead fish juveniles and water in the aquarium VS20 were used for EUS transmission.

Three aquaria holding 100 litres of clean, de-chlorinated tap water were set up. Striped snakehead juveniles were put in the tanks together with EUS-induced fish. Each tank received 1 EUS transmitter and 2 litres of water from aquarium VS20. The details are as follows:

- Aquarium A contained 15 virus-treated fish and was held in the temperature-controlled room at 20±0.5°C. The fish were survivors from experiment 4.3.2 (Chapter 4). They had been injected i/p with virus at 6.83 $\log_{10}$ $TCID_{50}$/0.1 ml/fish 3 months before the EUS transmission trial started. They appeared clinically normal.

- Aquarium B contained 25 non-treated fish held in the temperature-controlled room at 20±0.5°C. The fish came from the same stock as the fish in Chapter 4. They had been acclimated in the temperature-controlled room for 2 months and appeared in good health.
- Aquarium C contained 20 non-treated fish held in the wet laboratory inside the building at 28±1°C. The fish were from the same stock as the fish in Chapter 4. They had been acclimated in the wet laboratory for more than 3 months and appeared in good health.

Aquarium B was assigned as a control for aquarium A to compare the outcome between non-treated fish and virus-injected fish. Aquarium C was assigned as a control for aquarium B to compare the responses between fish held at 20±0.5°C and 28±1°C. Fish were fed daily and no aeration was given. Fifty litres of water were changed twice a week. Clinical signs and mortalities were observed. The experiment was terminated on day 14. No virus isolation was attempted as cell lines maintained in the laboratory were not very healthy. Time did not allow completion of virus isolation studies at AAHRI as period of stay in Thailand was coming to an end. Histological sections of tissue from 3 fish each in aquarium A and B were prepared at AAHRI and later, examined at the Institute of Aquaculture for mycotic granulomas in order to verify successful EUS transmission.

5.4 Results

5.4.1 EUS Induction in Striped Snakehead Fish Juvenile Using Rhabdovirus Isolate T9412 and *Aphanomyces invaderis* Strain RF-6 at 20±0.5°C and 29±2°C

Fish juveniles were given i/m and i/p inoculations with rhabdovirus isolate T9412 to ensure clinical changes. All groups of fish injected with either virus or L-15 medium developed a dark coloration at the i/m injection site on day 1. Some specimens showed a small opening in the muscle at i/m injection site. There was little difference between the 8 aquaria during the first few days post-injection except that the virus-injected fish developed a greater degree of change than the L-15-injected fish. The onset of clinical changes with virus infection alone took 10-14 days at 20°C (Table 5.2) and 4 days at 29°C (Table 5.3). *Aphanomyces* spores were introduced to attempt double-infection on day 3 and the fungus caused some effects in various degrees. Clinical changes and behaviour in fish are recorded in Table 5.2 and Table 5.3 and detailed as follows:

At 20±0.5°C, fish codes VS20 and V20:

Rhabdovirus infection caused irregular and sluggish swimming of some fish on day 4 and small ulcer at the i/m site on day 7 in VS20 and on day14 in V20. Fungus started to cause skin irritation in VS20 on day 10 which was not observed in V20. The small ulcers were severely progressive in VS20 and EUS lesions (haemorrhagic ulcers with fungal hyphae) were first seen in 4 fish on day 14. Five fish in V20 also developed small ulcers on day 14 which advanced to small haemorrhagic lesions on day 17 but

Table 5.2 Behaviour and clinical changes of snakehead fish juveniles infected with rhabdovirus isolate T9412 and *Aphanomyces invaderis* strain RF-6 at 20±0.5°C. Fish were injected via i/m and i/p routes with 50 µl virus/route (5.53 $\log_{10}$ $TCID_{50}$/fish). Fungus bath challenge dose was 10^3 spores/ml for 3 days.

Code	VS20	V20	LS20	L20
Day 0	**Inject virus**	**Inject virus**	**Inject L-15**	**Inject L-15**
Day 1	All fish developed dark coloration at i/m injection site. Some fish also had small opening at i/m injection site. Virus-injected fish developed more marked lesions.			
Day 2	Dark coloration almost disappeared. Fish behaviour returned to normal.			
Day 3	**Bath spores**	**Control**	**Bath spores**	**Control**
Day 4	Abnormal swimming. 4 fish with dark skin at injection site.	Abnormal swimming. 3 fish with dark skin at injection site.	2 fish with dark skin at injection site.	2 fish with dark skin at injection site.
Day 6	1 fish with dark skin.	Normal	2 fish with ulcer lesions. 1 fish with white cloud colour of skin.	Normal
Day 7	1 fish with ulcer lesion. Some fish started to develop lesions.	Normal	2 fish with ulcer lesions. 1 white cloud colour fish died.	Normal
Day 10	1 fish with ulcer lesions. Skin irritation.	A few fish developed dark skin at injection site.	2 fish with ulcer lesions.	Normal
Day 14	4 fish with EUS lesions. Skin irritation and reduced feeding.	5 fish with small ulcers. Normal feeding.	7 fish with EUS lesions (2 with severe EUS). Skin irritation but normal feeding.	Normal
Days 17-18	4 fish with EUS lesions (1 fish had severe EUS and haemorrhagic vent with fungus). Skin irritation and reduced feeding.	5 fish with small haemorrhagic lesions. Normal feeding.	7 fish with EUS lesions (2 with severe EUS still alive). Skin irritation but normal feeding.	Normal

Table 5.2 cont

Code	VS20°C	V20°C	LS20°C	L20°C
Days 21-22	1 fish died and 3 fish with severe EUS. Skin irritation and reduced feeding.	1 fish died and 4 fish still with small ulcer. Normal feeding.	2 fish died. 3 fish with EUS lesions on the side of body. 1 fish with EUS lesions at the vent. 1 fish with EUS lesions at caudal peduncle. Skin irritation but normal feeding.	Normal
Days 24-26	7 EUS fish (4 new EUS fish developed lesion at injection site).	Small ulcers on 3 fish healed and fish returned to normal. 1 fish with small ulcer.	1 out of 5 EUS fish showed a large ulcer while 4 fish started to recover. No skin irritation and normal feeding.	Normal
Day 28	2 fish died. 12 EUS fish present. All fish with severe skin irritation and no response to the feed.	1 fish with a small ulcer. 18 fish normal.	1 fish with severe EUS lesions. 2 fish with minor EUS lesions. 1 fish recovered. 15 fish normal.	Normal
Day 30	All 17 fish developed EUS with multiple lesions on the body including head. All fish had severe skin irritation and did not respond to the feed. Most fish floating near the water surface. The EUS-induced fish were taken for virus isolation, fungus isolation and histopathology.	1 fish remained with small ulcer. 18 fish normal. The fish were taken for virus isolation and histopathology.	1 fish with large EUS lesions. 1 fish with minor EUS lesion on body. 1 fish with minor EUS lesion at the vent. Rest of 15 fish normal. The fish were taken for virus isolation, fungus isolation and histopathology.	Normal Fish were taken for virus isolation and histology.
Virus isolation	20% (2/10)	20% (2/10)	0% (0/6)	0% (0/6)
Fungus isolation	Yes	not done	Yes	not done
Histopathology	Mycotic granulomas.	No mycotic granuloma.	Mycotic granulomas.	No mycotic granuloma

Table 5.3 Behaviour and clinical changes of snakehead fish juveniles infected with rhabdovirus isolate T9412 and *Aphanomyces invaderis* strain RF-6 at 29±2°C. Fish were injected via i/m and i/p routes with virus at 50 µl/route (5.53 $\log_{10}$ $TCID_{50}$/fish). Fungus bath challenge dose was 10^3 spores/ml for 3 days.

Code	VS29°C	V29°C	LS29°C	L29°C
Day 0	**Inject virus**	**Inject virus**	**Inject L-15**	**Inject L-15**
Day 1	All fish developed dark coloration at i/m injection site. Some fish also had small opening at i/m injection site. Virus-injected fish developed more marked lesions.			
Day 2	Dark coloration almost disappeared. Fish behaviour returned to normal.			
Day 3	**Bath spores**	**Control**	**Bath spores**	**Control**
Day 4	Slow movement	No dark skin. Some fish with petechial haemorrhages.	Normal	Normal
Day 6	Normal	Normal	Normal	Normal
Day 7	Some fish with small ulcers.	Normal	Normal	Normal
Day 10	Normal	Normal	Normal	Normal
Days 14-30	Normal The fish were taken for virus isolation and histology.	Normal The fish were taken for virus isolation and histology.	Normal The fish were taken for virus isolation and histology.	Normal The fish were taken for virus isolation and histology.
Virus isolation	0% (0/10)	0% (0/10)	0% (0/9)	0% (0/9)
Histopathology	No mycotic granuloma.	No mycotic granuloma.	No mycotic granuloma.	No mycotic granuloma

never progressed to EUS lesions. The first EUS-induced fish died on day 21 which was 7 days after establishment of EUS lesions on the body. While the condition of the fish in VS20 was deteriorating, those in V20 were improving. Four further fish in VS20 developed EUS on day 24-26, about 10 days after the first 4 EUS-induced fish had developed characteristic lesions. The total number of live fish with EUS lesions increased to 7 fish on day 26, 12 fish on day 28 and all 17 surviving fish on day 30. At the end, 3 EUS-induced fish had died and 17 live EUS-induced fish remained. Fish in VS20 started to feed less on day 14 and did not respond on day 28. Fish in VS20 encountered 2 periods of skin irritation at days 10-22 and days 28-30 but no external parasites were found in moribund specimens. In contrast, the lesions of fish in V20 started to heal on day 21-22 and only 1 dead fish was found on day 21. At the end of the experiment, only 1 fish in V20 remained with a small ulcer while 18 fish were of normal appearance. These findings indicate that the combination of rhabdovirus and *Aphanomyces* fungus can induce severe EUS disease condition in juvenile snakehead fish at 20°C.

At 20±0.5°C, fish codes LS20 and L20:

After infection with RF-6 spores, 2 fish in LS20 showing a darkened coloration at the i/m injection site developed small lesions on day 6. These small lesions grew larger to form ulcers on day10, but no fungal hyphae were seen on the ulcer surface. Many clinical changes were observed in fish group LS20, while the control fish group L20 remained normal thought 30 days observation.

Aphanomyces continued to cause damage to the fish and major clinical changes were dominated by this infection. On day 6, 1 fish died without ulcerative lesions but with a white cloud color skin. On day14, 5 fish with moderate EUS lesions (shallow ulcers with fungus hyphae) first appeared and showed skin irritation while 2 additional fish had severe EUS lesions. The 2 severely infected fish died on day 21. Interestingly, on day 24, the remaining 5 EUS-induced fish started to recover and signs of skin irritation disappeared. At the end of the experiment on day 30, 1 fish remained with severe EUS lesions, 2 fish with minor lesions (small ulcers and fungal hyphae) and 15 fish were of normal appearance. These findings indicate that the *Aphanomyces* fungus alone can induce EUS symptom in juvenile snakehead fish at 20°C but with limited severity.

At 29±2°C, fish codes VS29, V29, LS29 and L29:

Rhabdovirus infection caused lethargy and petechial haemorrhages in juvenile snakehead fish on day 4 at 29°C. This behaviour and the clinical changes had gone by day 6. Nevertheless, some fish in group VS29 which had been double-infected with virus and RF-6 spores, seemed to develop small lesions at the i/m injection site on day 7 similar to those which occurred about the same time in fish groups VS20 and LS20. The small lesion did not progress further and had healed with the fish returning to normal appearance by day 10. Fish in both groups VS29 and V29 remained healthy and of normal appearance through to the end of the experiment. These findings indicate that the combination of rhabdovirus and *Aphanomyces* fungus can not induce EUS in juvenile snakehead fish at 29°C.

Fish in group LS29 were not affected by exposure to RF-6 spores alone. After day 4, both fish groups LS29 and L29 were normal through to the end of the experiment. These findings indicate that the *Aphanomyces* fungus alone can not induce EUS in juvenile snakehead fish at 29°C.

Rhabdovirus re-isolation:

At the end of the experiment, rhabdovirus could be re-isolated from only 20% (2/10) of fish in groups VS20 and V20 (Table 5.4). On initial inoculation of 5 tissue extract samples from 10 fish of VS20 (2 fish/sample), BF-2 cells gave 1 positive CPE on day 7, 1 doubtful CPE on day 10 and 3 negative CPE. At the first passage, BF-2 cells gave only 1 positive CPE as did HCK cells. One sample from fish group V20 also showed 1 positive CPE in both BF-2 and HCK cells. There was no recovery of virus from 6 fish of LS20, 6 fish of L20, 10 fish of VS29, 10 fish of V29, 9 fish of LS29 or 9 fish of L29. No antiserum was available against isolate T9412, so serological confirmation of identity could not be carried out. However, the rounding-up type CPE began to be observed on day 7 in BF-2 cells and on day 10 in HCK cells following initial inoculation. The CPE was found to develop faster on first passage at day 3 in BF-2 cells and day 5 in HCK cells.

***Aphanomyces* pathogen re-isolation:**

Two EUS-induced fish were selected for fungus isolation, 1 from group VS20 and 1 from group LS20. *Aphanomyces* was successfully isolated from small pieces of muscle tissue beneath the lesions. After incubating a few pieces of tissue in glucose-peptone broth for 2 h, fungal hyphae started to produce new viable hyphal tips (Figure 5.1a).

Table 5.4 Observation of CPE and percent virus isolation from striped snakehead fish juveniles surviving induced EUS. Tissue extracts (1:50 dilution) were simultaneously inoculated into cell cultures and incubated at 23°C. Controls cells were inoculated with L-15 medium.

Code & cell line	Culture passage	Virus isolation from internal organs of snakehead fish surviving from EUS induction. (tissue extract sample numbers)											Cell control
		1	2	3	4	5	6	7	8	9	10	11	12
VS20		20% virus isolation from 10 fish											
HCK	ini	–	–	–	?	–							–
	1 st	–	–	–	+	–				ns			–
BF-2	ini	–	?	–	+	–							–
	1 st	–	–	–	+	–							–
V20		20% virus isolation from 10 fish											
HCK	ini	+	–	–	–	–							–
	1 st	+	–	–	–	–				ns			–
BF-2	ini	+	–	?	–	?							–
	1 st	+	–	–	–	–							–
LS20		0% virus isolation from 6 fish											
HCK	ini	–	–	–									–
	1 st	–	–	–						ns			–
BF-2	ini	–	–	–									–
	1 st	–	–	–									–
L20		0% virus isolation from 6 fish											
HCK	ini	–	–	–									–
	1 st	–	–	–						ns			–
BF-2	ini	–	–	–									–
	1 st	–	–	–									–
VS29		0% virus isolation from 10 fish											
HCK	ini	–	–	–	–	–	–	–	–	–	–		–
	1 st	–	–	–	–	–	–	–	–	–	–		–
BF-2	ini	–	–	–	–	–	–	–	–	–	–	ns	–
	1 st	–	–	–	–	–	–	–	–	–	–		–
V29		0% virus isolation from 10 fish											
HCK	ini	–	–	–	–	–	–	–	–	–	–		–
	1 st	–	–	–	–	–	–	–	–	–	–		–
BF-2	ini	–	–	–	–	–	–	–	–	–	–	ns	–
	1 st	–	–	–	–	–	–	–	–	–	–		–
LS29		0% virus isolation from 9 fish											
HCK	ini	–	–	–									–
	1 st	–	–	–						ns			–
BF-2	ini	–	–	–									–
	1 st	–	–	–									–
L29		0% virus isolation from 9 fish											
HCK	ini	–	–	–									–
	1 st	–	–	–						ns			–
BF-2	ini	–	–	–									–
	1 st	–	–	–									–

ns = no samples

At 4 h incubation, the hyphae had grown longer and become more branched. The selected pieces of tissue with viable fungal hyphae were placed on GPY agar plates and incubated at 20±0.5°C. On day 1, fungal hyphae had grown and penetrated into the agar and many new branches were produced (Figure 5.1b). The hyphae then started growing away from the centre and faster than the growth of bacteria. On day 2, material was subcultured to a new GPY agar plate to remove contaminating bacteria (Figure 5.1c). Two further subcultures on GPY agar were performed and pure cultures of *Aphanomyces* fungus were obtained. There was no attempt to test fungus growth at 37-38°C but the successful isolation of the fungus from small pieces of muscle tissue beneath the lesions indicated recovery of the *Aphanomyces* pathogen.

Macroscopic and microscopic changes:

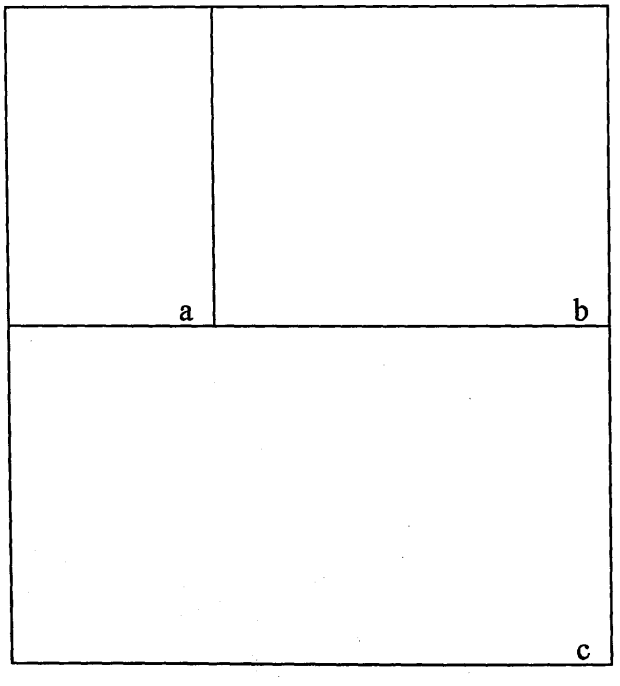
Clinical changes in EUS-induced fish were assessed macroscopically and microscopically at the end of the experiment. Severe macroscopic changes were found in fish challenged with both rhabdovirus and fungus at 20°C (code VS20). Most fish had multiple EUS lesions located mainly towards the posterior part of the body (Figure 5.2a and 5.2b). In contrast, control fish challenged with rhabdovirus only and held at 20°C (code V20) appeared macroscopically normal (Figure 5.3) except for 1 of 19 fish which retained a small area of scale damage. Most fish challenged with fungal spores only and held at 20°C (code LS20) also appeared normal but a few specimens retained EUS lesions on the body (Figure 5.4). Fish in the other 5 aquaria at 20°C and 29°C all appeared normal.

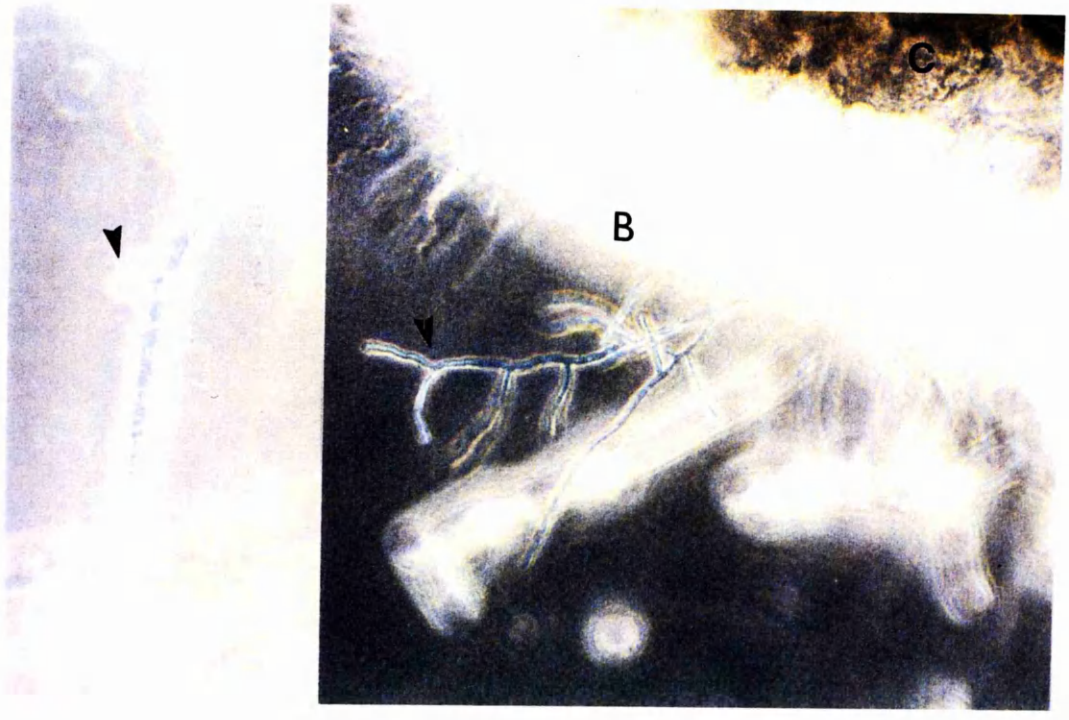
Figure 5.1 *Aphanomyces* re-isolation from muscle tissue of EUS-induced snakehead fish juvenile.

a. 2 h in glucose-peptone broth. New fungal hyphae starting to growth (arrow). Magnification ×130

b. One day after transfer of a fragment of tissue with viable fungal hyphae on to glucose-peptone agar. More fungal hyphae were produced (arrow) and grew out from the centre (C) faster than the growth of bacteria. The white cloud area indicates zone of bacterial contamination (B). Magnification ×50

c. Two days after subculture to a new agar plate. Viable branched and non-septate fungal hyphal were obtained with less bacterial contamination. Magnification ×50




B
C

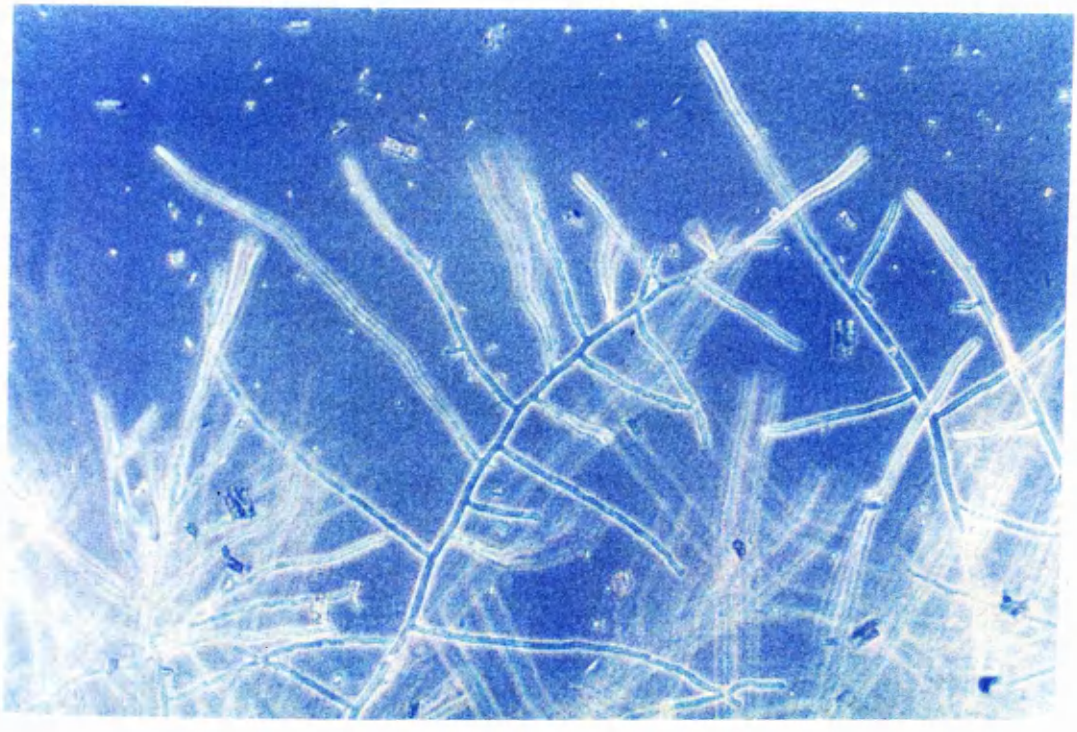


Figure 5.2 Fish infected with rhabdovirus T9412 and fungal spores RF-6 at 20°C. Multiple EUS lesions on the fish body on day 30 post-infection. The ulcers were normally white to cream in color at the centre and pink at the edges. Severe haemorrhagic edges to the ulcers just occurred when the fish were caught.

a. Dorsal view

b. Lateral view

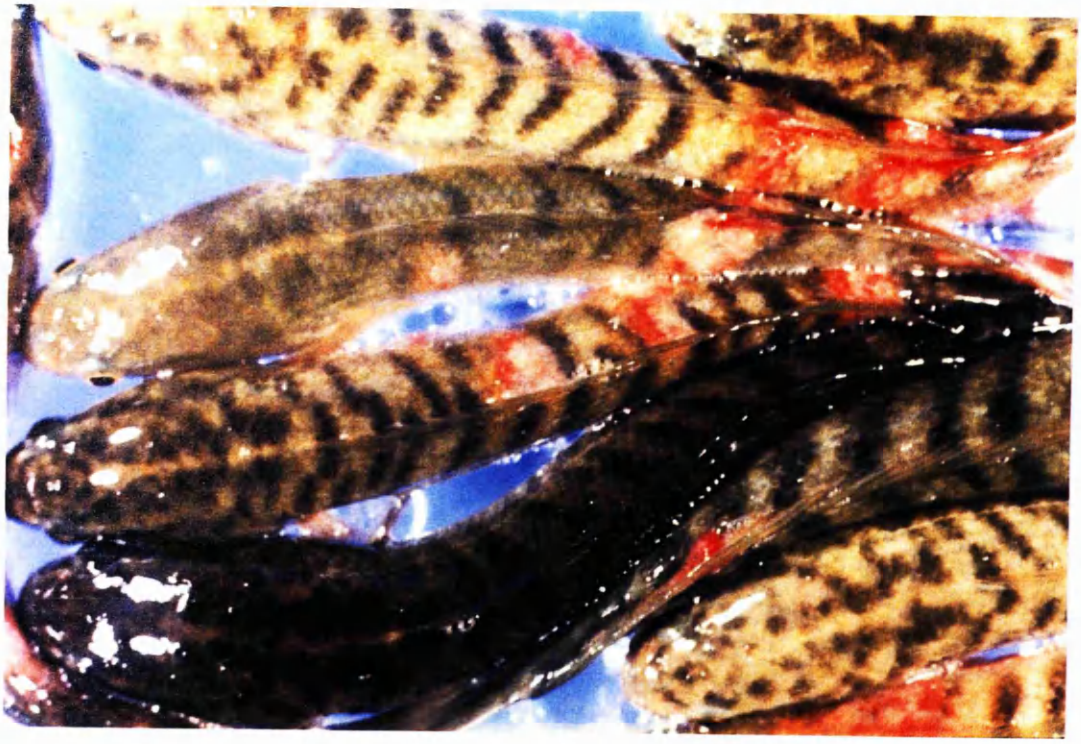
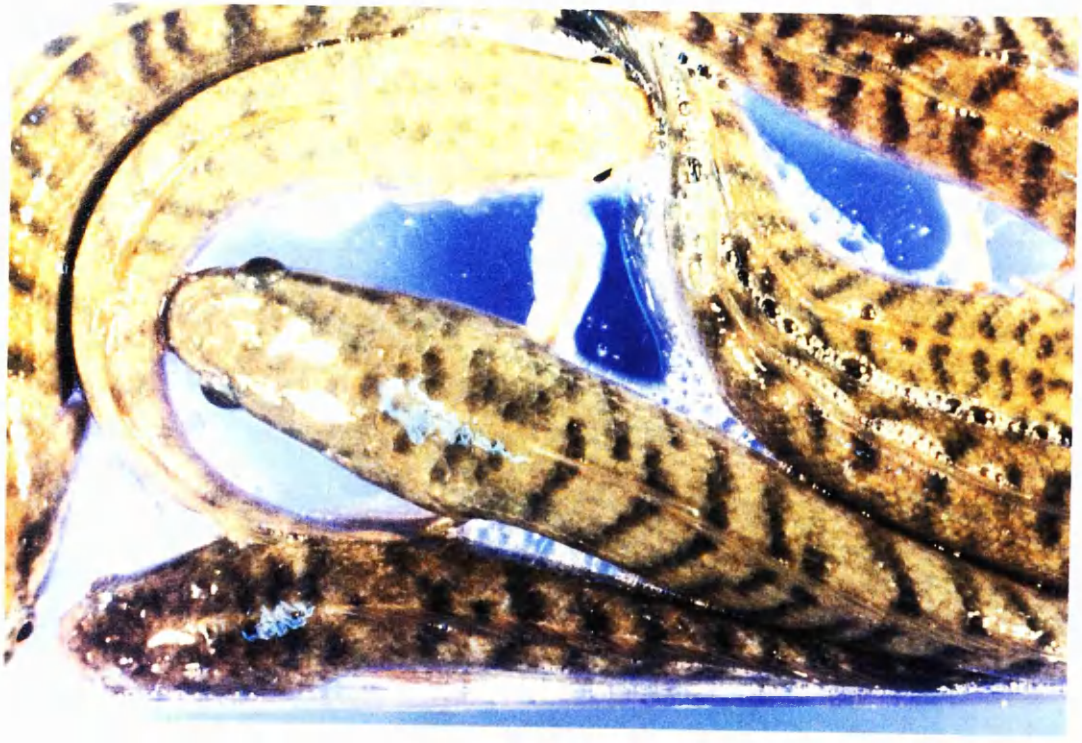
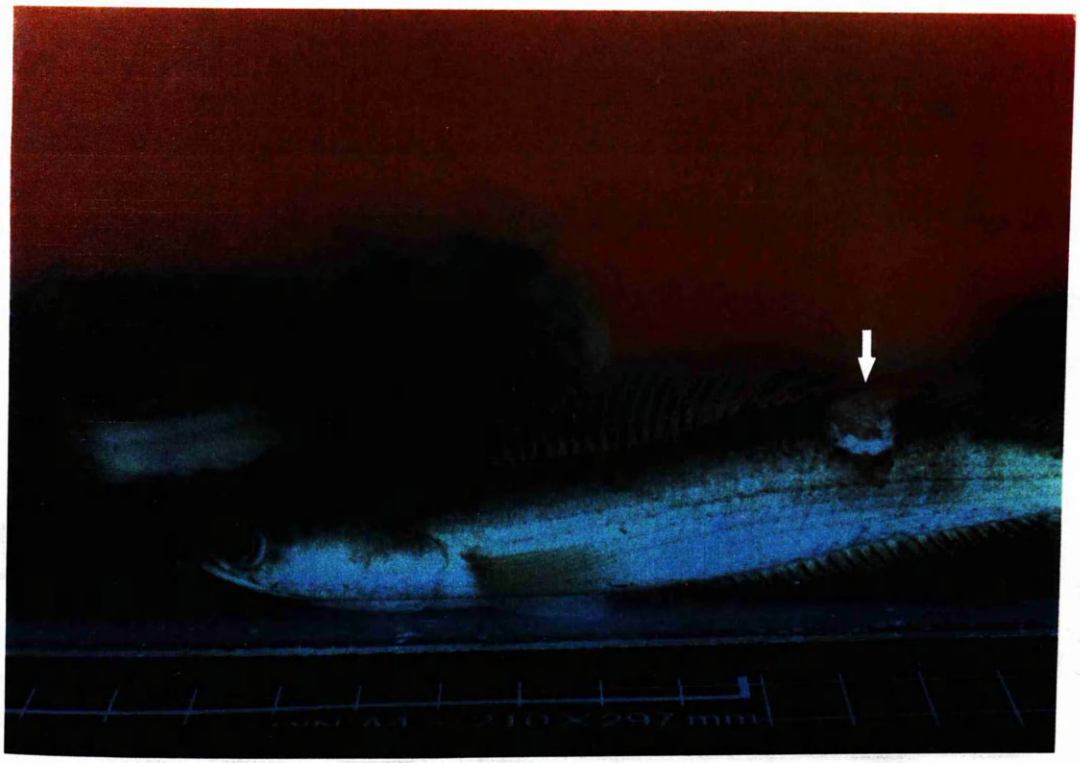


Figure 5.3 Fish infected with rhabdovirus alone and held at 20°C. Fish appeared normal and no EUS lesions were observed on day 30 post-infection.

Figure 5.4 Fish injected with L-15 cell culture medium and bathed with RF-6 spores at 20°C. A few showed EUS lesions on the body and 1 fish had severe ulcerative lesions with fungal hyphae (arrow).



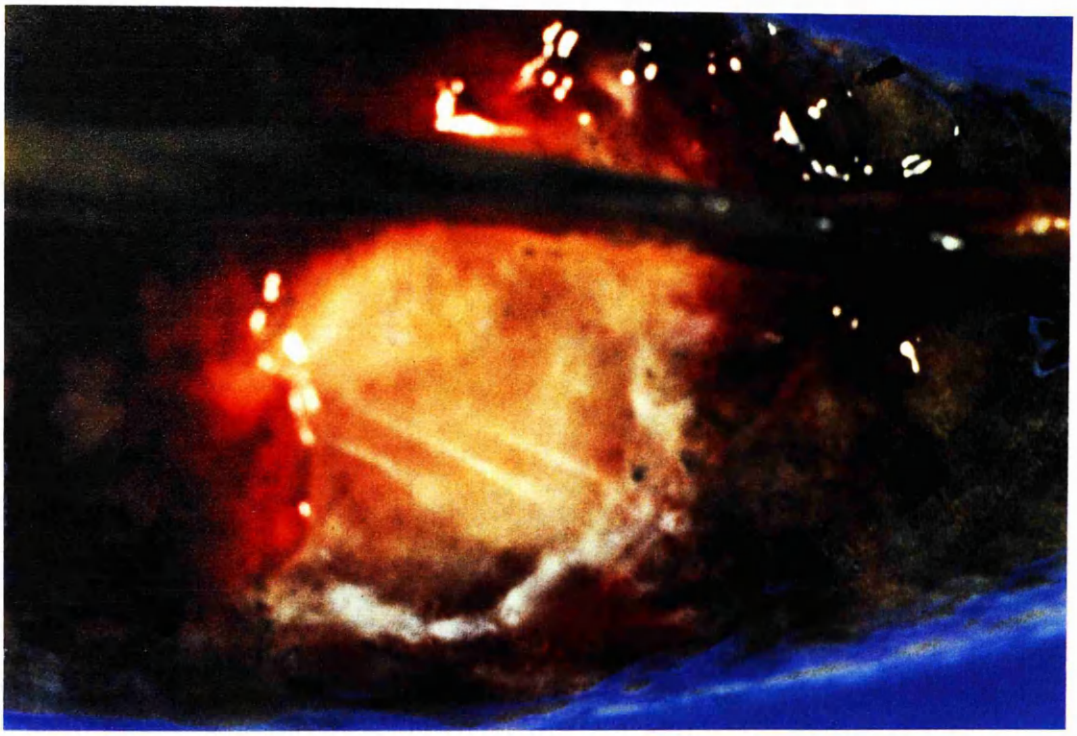


EUS lesions from the induction experiment developed as ulcerative lesions with fungal hyphae (Figures 5.5 and 5.6). When the EUS-induced fish were left undisturbed in the aquaria the ulcers were white to pink in colour (low degree of haemorrhage) and fungal hyphae protruded from the surfaces. When the fish were caught in a net, they became excited and the ulcer margins turned red in colour. Beneath the ulcers, some degree of focal haemorrhage was observed and more severe haemorrhages were found with prolonged infections (Figure 5.7). A few fish with lesions on the head or ventral part of the body were also found (Figure 5.8). The internal organs of EUS-induced fish were generally normal but fish with lesions appearing on abdomen or vent had some degree of haemorrhage or congestion in the internal organs especially the liver.

Microscopic observation of the ulcer surfaces revealed the presence of a typical *Aphanomyces* fungus with non-septate mycelium and reproductive structure, spore ball, in wet smears (Figures 5.9-5.10). There were no fish parasites on the ulcer surfaces or gills. Successful EUS induction in the laboratory was also confirmed by the presence of mycotic granulomas in histological sections of muscle tissue. The mycotic granulomas were found only in the fish in groups VS20 and LS20 held at 20°C. There was no sign of mycotic granulomas in the others 6 groups of fish. Cross-sections through the body of control fish code L20 showed the typical structure of skeletal muscle of scale fish (Figure 5.11). Sections through the severe ulcers (Figure 5.7) of fish code LS20 bathed with *Aphanomyces* spores showed severe necrosis of muscle, loss of epidermis, dermis and scales (Figure 5.12) and invasion by fungal hyphae with a low degree of haemorrhage except at the edge of the ulcer (Figure 5.13).

Figure 5.5 Higher magnification of Figure 5.4 showing a classic EUS lesion with necrotic muscle tissue and low degree of haemorrhage at the center of the ulcer. The hair-like structure of the fungal hyphae projecting from the ulcer surface were seen only when the fish was in the water.

Figure 5.6 Higher magnification of the affected fish in Figure 5.2 showing haemorrhage at the edge of the ulcer and hair-like structure of the fungal hyphae covering the ulcer.



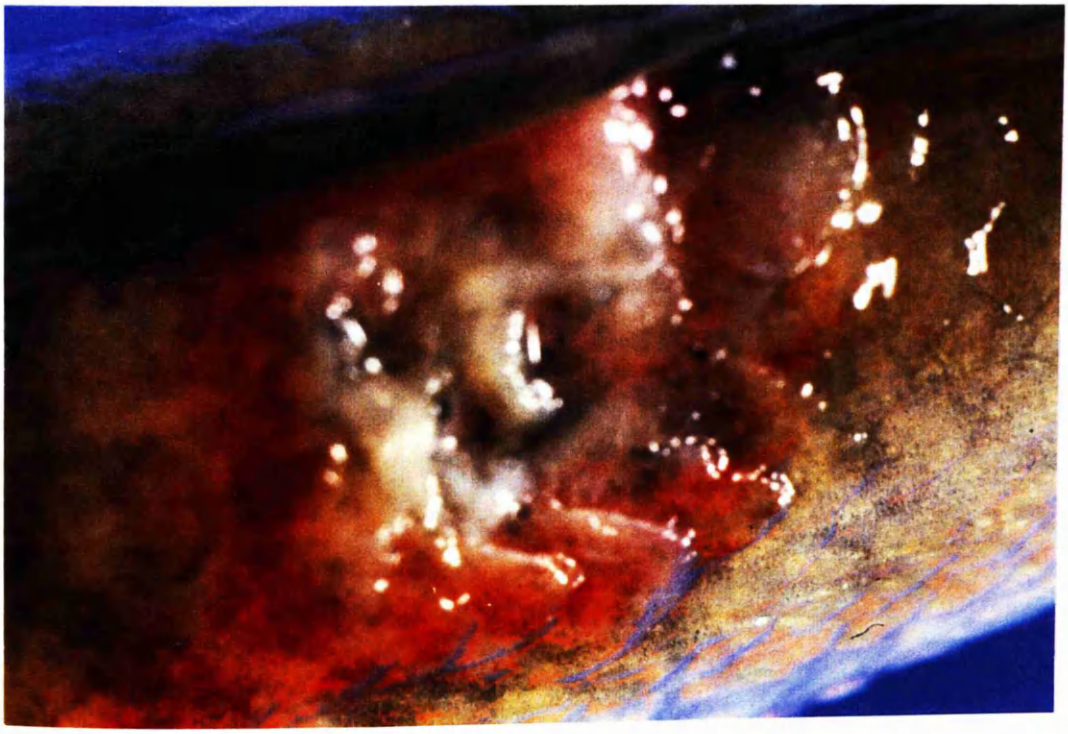
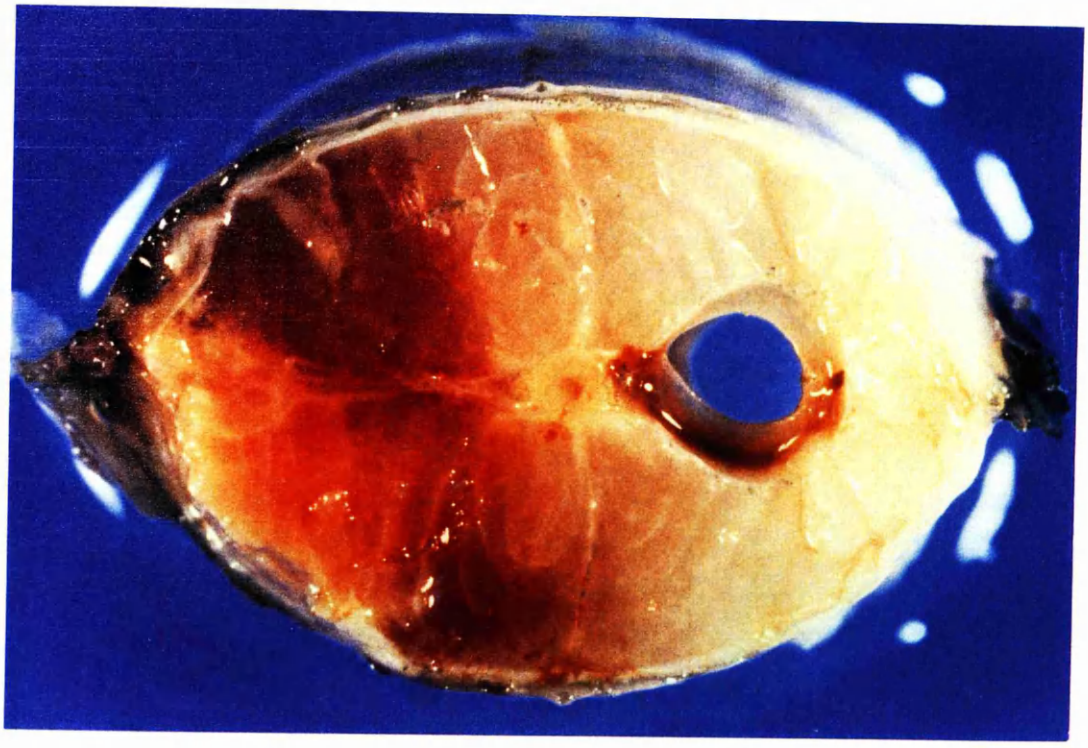


Figure 5.7 Cross section of the fish body through the ulcer showing a spread of damaged tissue with haemorrhage in the fish body beneath the shallow ulcer.

Figure 5.8 EUS-induced fish showing a lesion with fungal hyphae on the head close to the operculum and a lesion around the vent with a large area of haemorrhage.



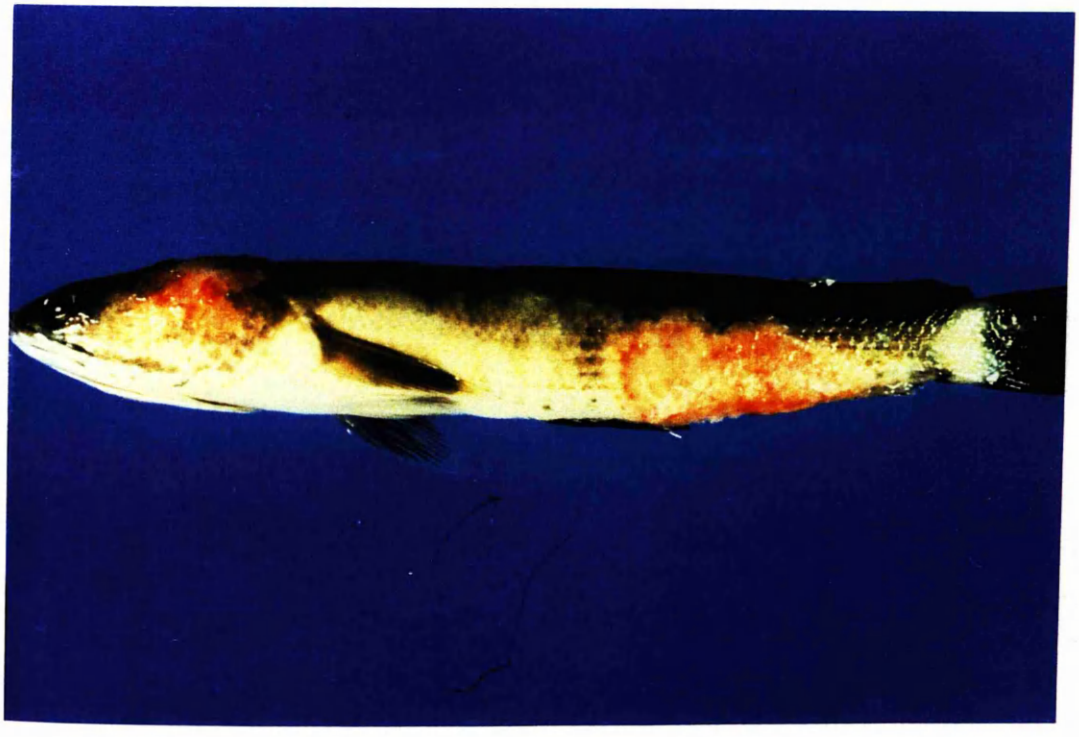
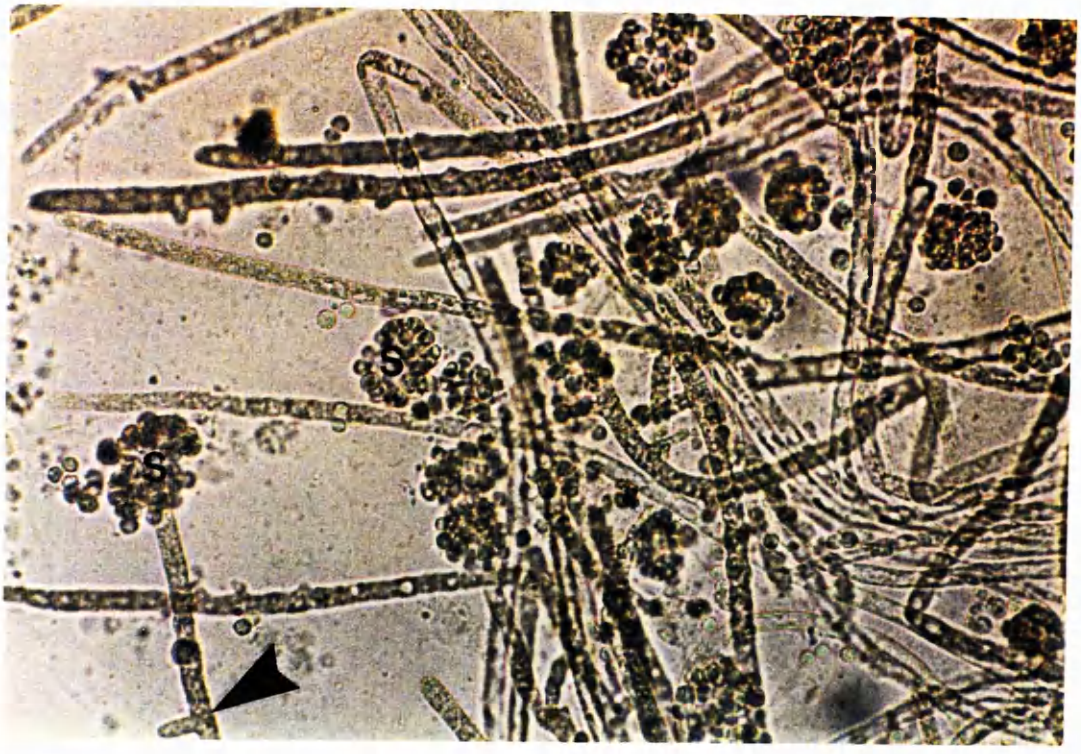


Figure 5.9 Wet smear of lesion surface from EUS-induced fish showing branched and aseptate hyphae (arrow) and sporangia (S) of *Aphanomyces* fungus. Magnification ×130

Figure 5.10 Sporangia (S) was a part of reproductive structure of *Aphanomyces* fungus from which the free-swimming zoospores were released. Magnification ×260


S
S

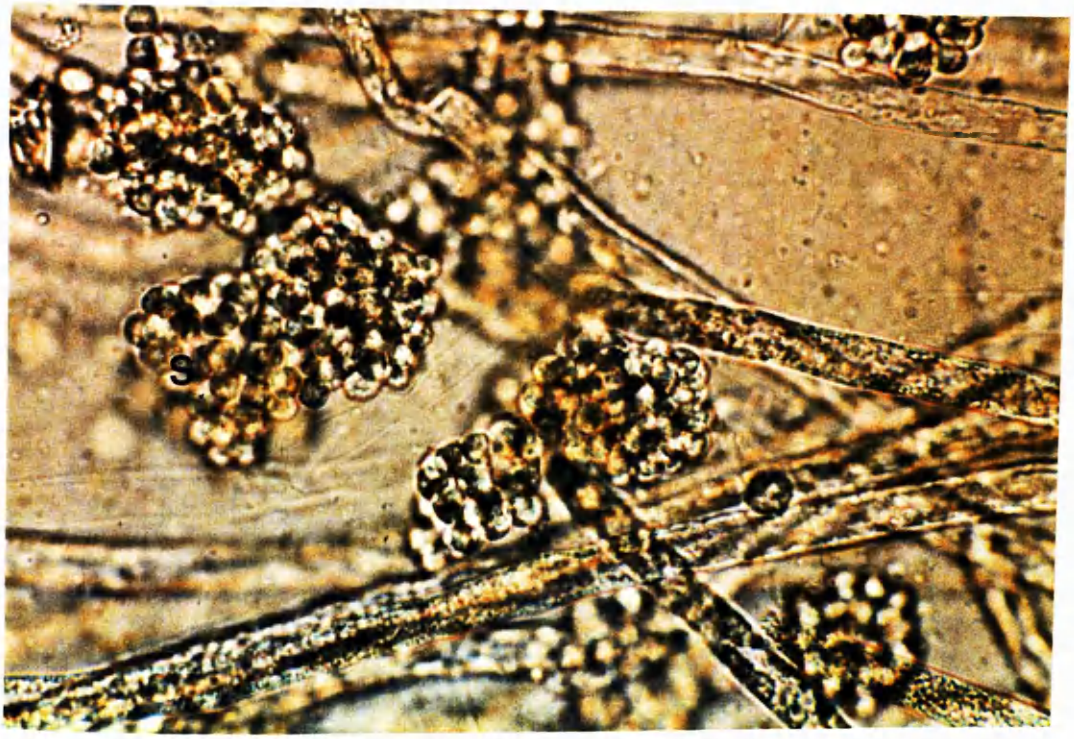
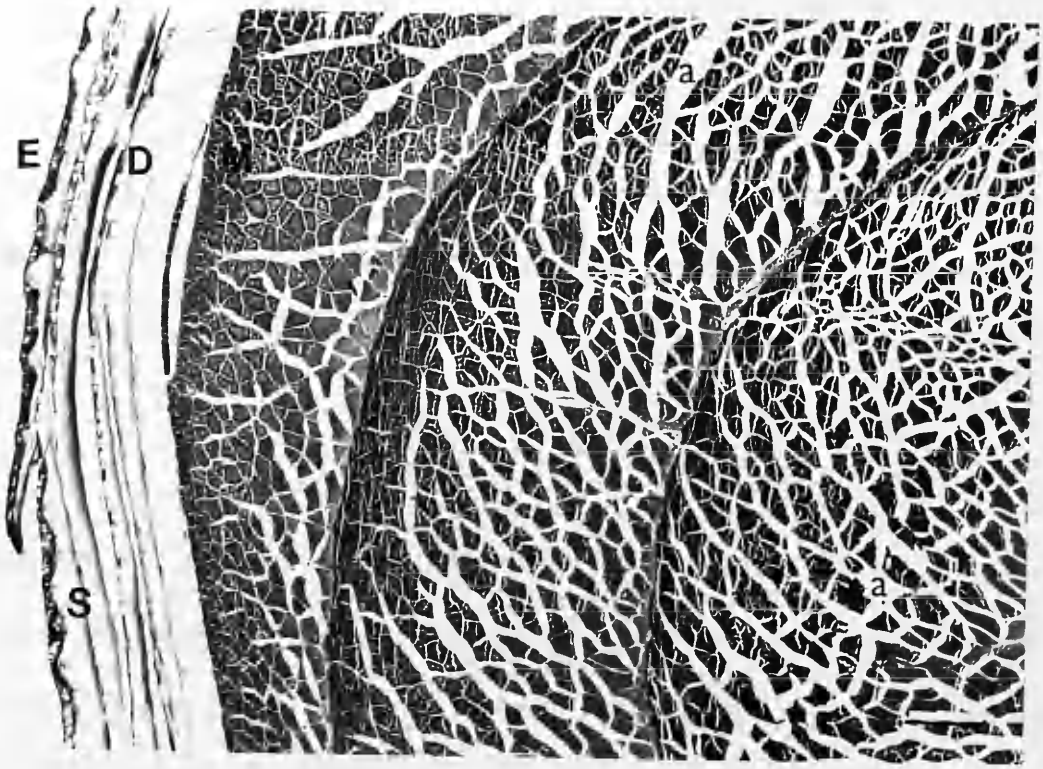
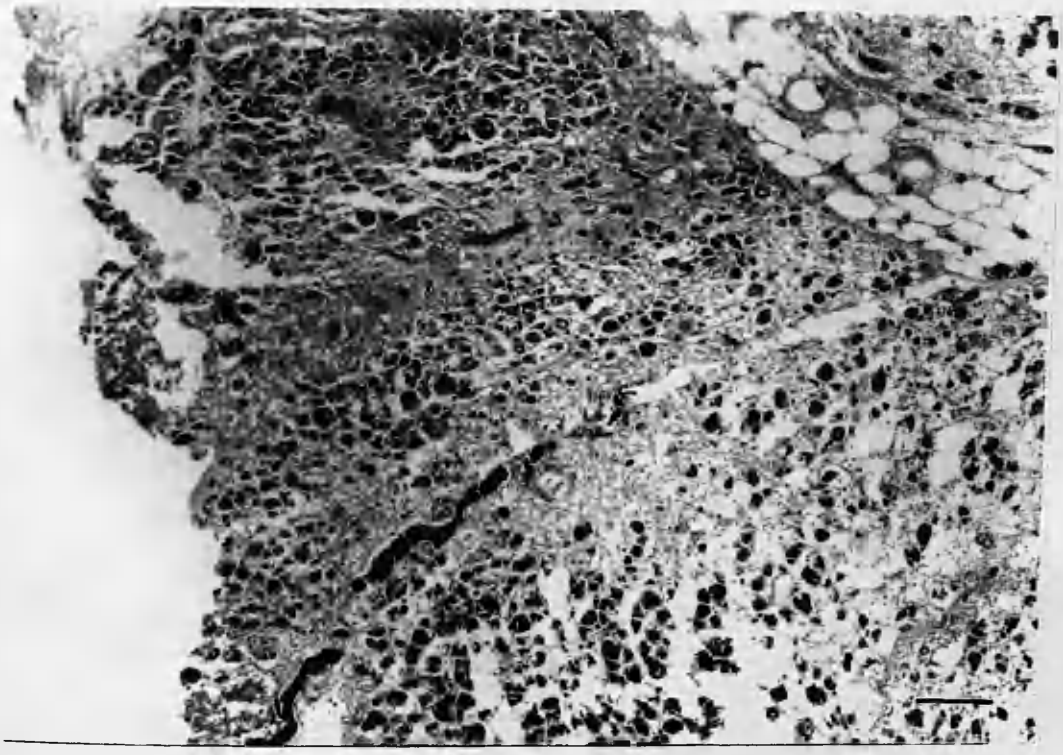

S

Figure 5.11 Control fish injected with L-15 medium only and held at 20°C for 30 days showing typical structure of epidermis (E), dermis (D), muscle tissue (M) and scales (S). a = artefact from tissue preparation, H&E, Bar = 205 μm

Figure 5.12 Section through ulcer (Figure 5.7) of fish injected with L-15 and bathed with fungal spores RF-6 showing an open lesion, loss of epidermis, dermis and part of muscle tissue, severe muscle necrosis, low degree of haemorrhage, invasion of fungal hyphae and mycotic granulomas. H&E, Bar = 205 μm


E
D
S
a
a

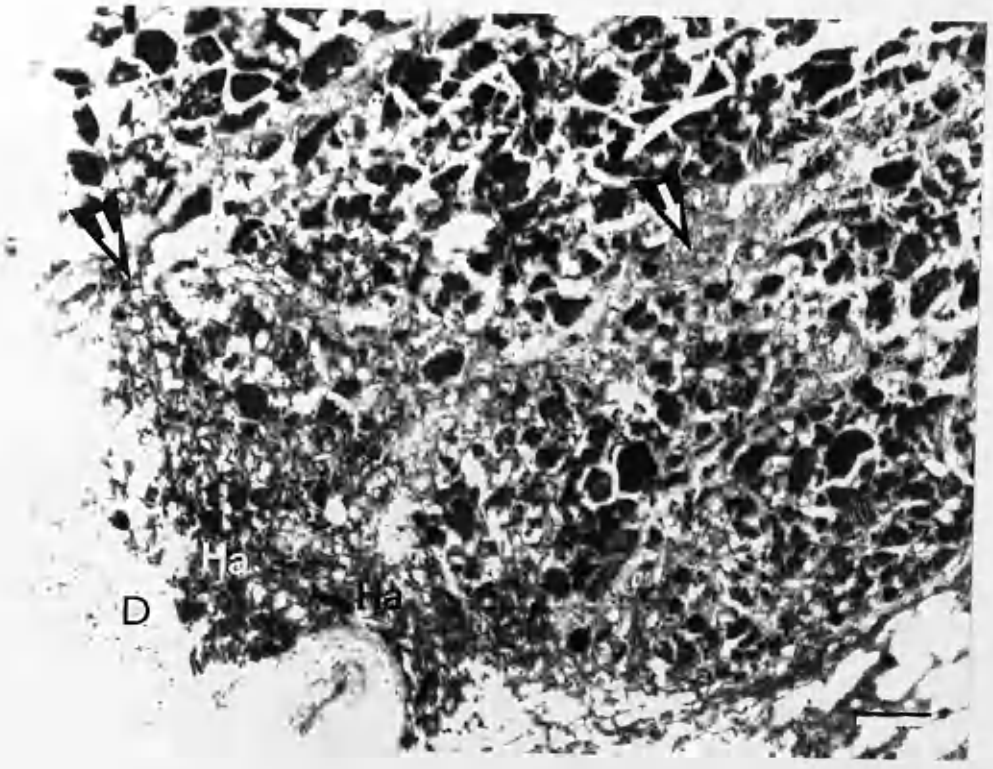


Sections through the ventro-lateral lesions of fish code VS20 injected with rhabdovirus and bathed with *Aphanomyces* spores exhibited severe invasion with fungal hyphae. The fungal hyphae invaded skeletal muscle and reached the peritoneal cavity (Figure 5.14-5.16) but signs of haemorrhage were rare. The fungal hyphae could invade inside or outside muscle bundles and cause necrosis of nearby muscle bundles. The fungus was then encapsulated by epitheloid cells and formed granulomas. Average diameter of fungal hyphae in the muscular layer was 16.4 ± 1.1 μm (mean ± SE, n = 8).

There were no significant changes in the internal organs of any experimental group. A few fish in each group had a low degree of histopathological change such as hepato-pancreas necrosis, endocrine pancreas necrosis, hepatic necrosis, liver vacuolation, liver congestion or kidney tubule necrosis.

Figure 5.13 Closer view of the edge of the ulcer of Figure 5.12 showing loss of dermis (D) and part of muscle tissue and severe invasion of fungal hyphae (arrows) in the muscle with limited cellular response. Severe haemorrhage (Ha) at the edge of the ulcer likely caused by frightening the fish with a catching net. H&E, Bar = 82 μm

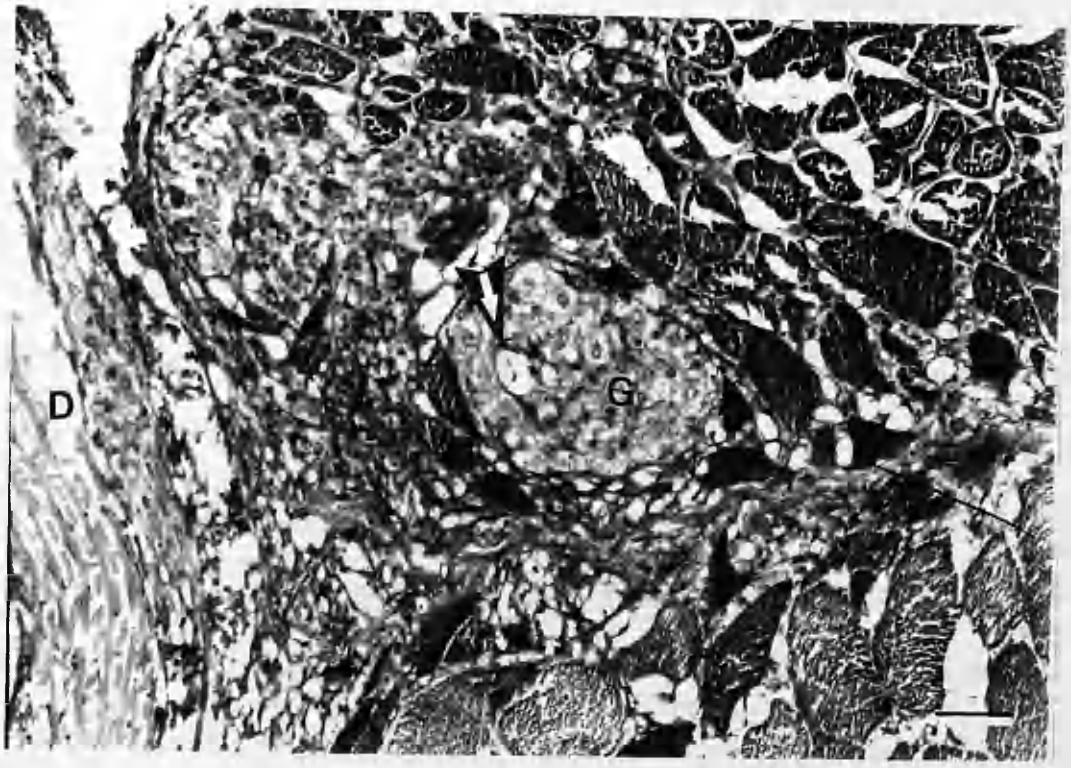
Figure 5.14 Ventro-lateral lesion of snakehead fish injected with rhabdovirus, bathed with RF-6 spores and held at 20°C for 30 days. Loss of epidermis, scales and part of dermis (D). Fungal hyphae penetrating deeply into abdominal cavity (Ab). Myonecrosis and low degree of haemorrhage were observed. H&E, Bar = 205 μm

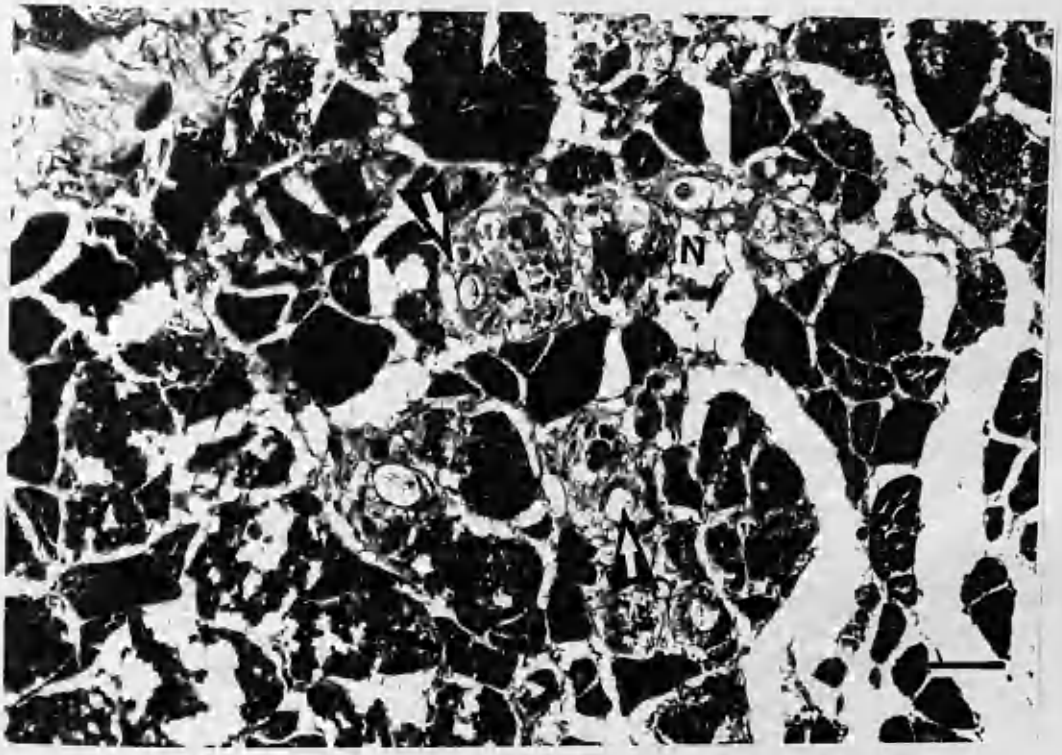

Ha
D
Ha


1
D
2
Ab

Figure 5.15 Closer view of area 1 in Figure 5.14. Fungal hyphae (arrow) surrounded by epithelioid cells to form granuloma (G) near dermis (D) with low degree of haemorrhage. H&E, Bar = 40 μm

Figure 5.16 Closer view of area 2 in Figure 5.14. Myonecrosis (N) in skeleton muscle caused by invasion of mass of fungal hyphae (arrows). Average diameter of fungal hyphae was 16.4 ± 1.1 μm (mean ± SE). H&E, Bar = 40 μm


D
G


N

5.4.2 EUS Transmission in Striped Snakehead Fish by Co-habitation With EUS-induced Fish at 20±0.5°C and 28±1°C

For the first 5 days following co-habitation, snakehead fish juveniles in all 3 aquaria remained normal. EUS fish transmitters were found dead on day 5 in every aquarium. During the following days, EUS began to develop (Table 5.5). The fish pre-treated with virus in aquarium A were more severely affected by the disease than the non-treated fish in aquarium B at 20°C. On day 8, fish with EUS lesions were first observed in 2 fish in aquarium A. The disease condition developed further and involved all 15 fish. Five fish died and 10 fish showed multiple lesions with severe fungal infection on day 14 (Figure 5.17). For aquarium B, 1 fish with EUS lesions was first seen on day 9 and 7 more fish suffering EUS lesions were noted on day 11 which increased to 21 fish by day 13. Two severely affected fish died during the observation period. Some affected fish seemed to heal and at the end of the experiment on day 14, 8 fish appeared normal but 15 fish still carried EUS lesions (Figure 5.18) with a clinical picture similar to that observed in the induction experiment.

Fish held at the higher incubation temperature of 28°C developed clinical signs of disease faster than fish held at 20°C as 7 fish in aquarium C showed minor lesions with fungal hyphae on day 8. Although initial transmission of EUS occurred rapidly with fish in aquarium C, the spread of disease within the aquarium was limited and only 8 out of 20 fish were affected. Furthermore, only 2 of the affected fish died while the

Table 5.5 Behaviour and clinical changes of snakehead fish juveniles caused by EUS transmitters. The transmitter, 1 EUS-induced striped snakehead with 2 litres of infected water, were distributed to 3 aquaria at 20±0.5°C or 28±1°C.

Code: Days	Aquarium A: 15 pre-treated fish and EUS transmitter (20°C)	Aquarium B: 25 non-treated fish and EUS transmitter (20°C)	Aquarium C: 20 non-treated fish and EUS transmitter (28°C)
Day 2	Fish were normal and fed well. The EUS-induced fish was alive.	Fish were normal and fed well. The EUS-induced fish was alive.	Fish were normal and fed well. The EUS-induced fish was alive
Day 5	The EUS-induced fish died.	The EUS-induced fish died.	The EUS-induced fish died.
Day 6	Fish were normal and fed well.	Fish were normal and fed well.	1 fish with skin damage. The rest were normal.
Day 8	2 fish with ulcers and fungal hyphae.	Fish still normal.	7 fish with minor ulcers and fungal hyphae.
Day 9	2 fish with severe ulcers and fungal hyphae.	1 fish with red ulcers and fungal hyphae. Some fish with skin irritation.	1 fish died. 6 fish with minor ulcers and fungal hyphae.
Day 11	1 fish died. 1 fish with severe ulcers and fungal hyphae. 13 fish with skin irritation and starting to develop disease. Fish did not respond to the feed.	1 fish with severe ulcers and fungal hyphae. 7 fish with minor ulcers and fungal hyphae. Some affected fish fed well. 17 fish were normal.	1 fish died. 6 fish with minor ulcers and little fungal hyphae. Some fish fed well.
Day 12	1 fish died. 13 fish with more advanced ulcers and fungal hyphae.	1 fish died. 7 fish with ulcers and fungal hyphae. 13 fish starting to develop disease. 4 fish still normal.	5 fish with minor ulcers and little fungal hyphae. 1 fish recovered. Fish fed well.
Day 13	1 fish died. 12 fish with ulcers and heavy fungal infection.	1 fish died. 4 fish with large ulcers and fungal hyphae. 15 fish with minor ulcers and fungal hyphae. 4 fish still normal.	The affected fish starting to recover. Fish fed well.
Day 14	2 fish died. 10 fish with ulcers and heavy fungal infection. 8 of them were fixed in 10% formalin for histopathology.	15 fish with ulcers and fungal hyphae. 11 of them were fixed in 10% formalin for histopathology. 8 fish normal and fed well.	18 fish normal and fed well.

Figure 5.17 EUS transmission in rhabdovirus-treated snakehead fish at 20°C. EUS lesions were shallow ulcers with little or no haemorrhage. The white cloudy discoloration on the fish body represented patches of fungal hyphae.

Figure 5.18 EUS transmission in non-treated snakehead fish at 20°C. Most EUS lesions with haemorrhgic edge and fungal hyphae developed towards posterior part of the body.

other 6 were able to recover. All 18 surviving fish appeared normal at the end of the experiment.

Microscopic examination of the ulcer surfaces revealed a mass of non-septate fungal hyphae, sporangia and spore balls in wet smears. There were no fish parasites on the ulcer surfaces or gills except rotifers and paramecium. EUS transmission in rhabdovirus-treated fish in aquarium A and non-treated fish in aquarium B on day14 at 20°C was proven by the presence of mycotic granulomas in histological sections of affected tissue. *Aphanomyces* fungal hyphae were able to invade fish tissue starting from the epidermis and penetrating to dermis, hypodermis and muscle tissue. Figure 19 shows the early invasion of the fungal hyphae in non-treated fish with hyperplasia of the epidermis and dermis. At a more advanced stage of invasion (Figure 5.20), scales and epidermis were eroded away and the fungal hyphae had penetrated deep into the muscle. Many mycotic granulomas were found in the muscle tissue of non-treated fish and rhabdovirus-treated fish (Figures 5.21, 5.22). Average diameter of fungal hyphae in the epidermis and dermis was 14.3 ± 0.4 μm (mean ± SE, n = 19) but in the muscle it was 16.4 ± 0.8 μm (mean ± SE, n = 4). There were no significant changes in the internal organs of both rhabdovirus-treated and non-treated groups of snakehead fish.

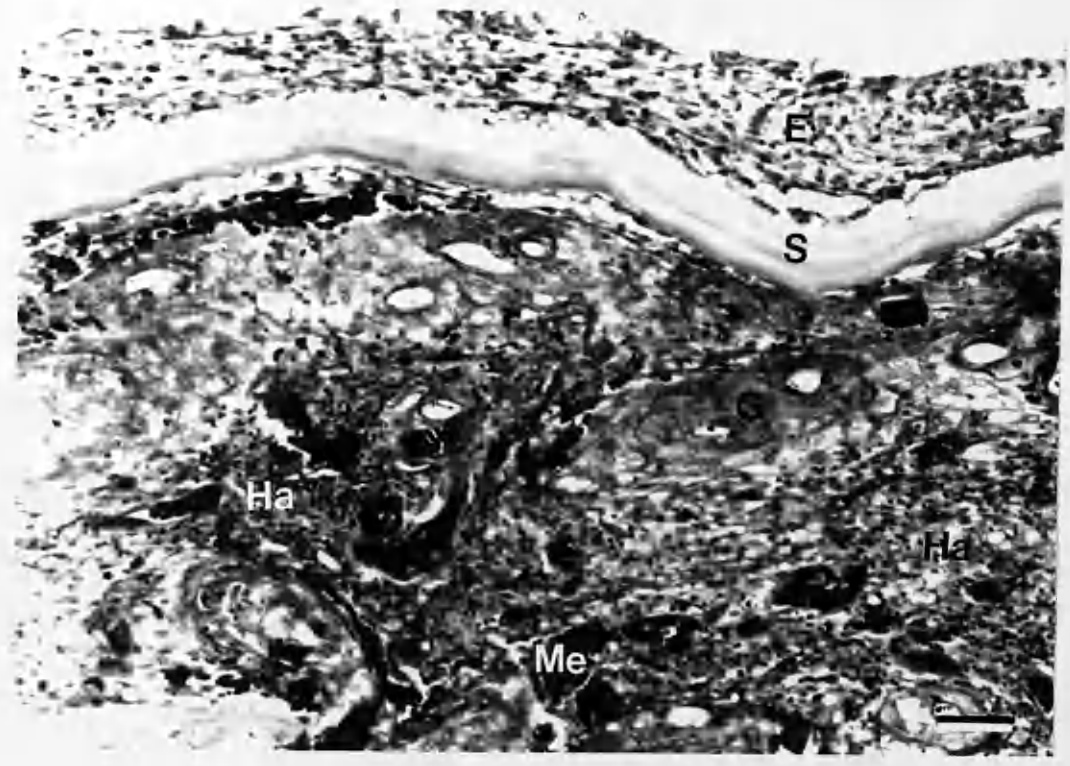
Figure 5.19 Early attachment and invasion of fungal hyphae (arrow) in epidermis (E) and dermis (D). Hyperplasia was observed in both layers. The muscle was normal. S = scale, H&E, Bar = 82 μm

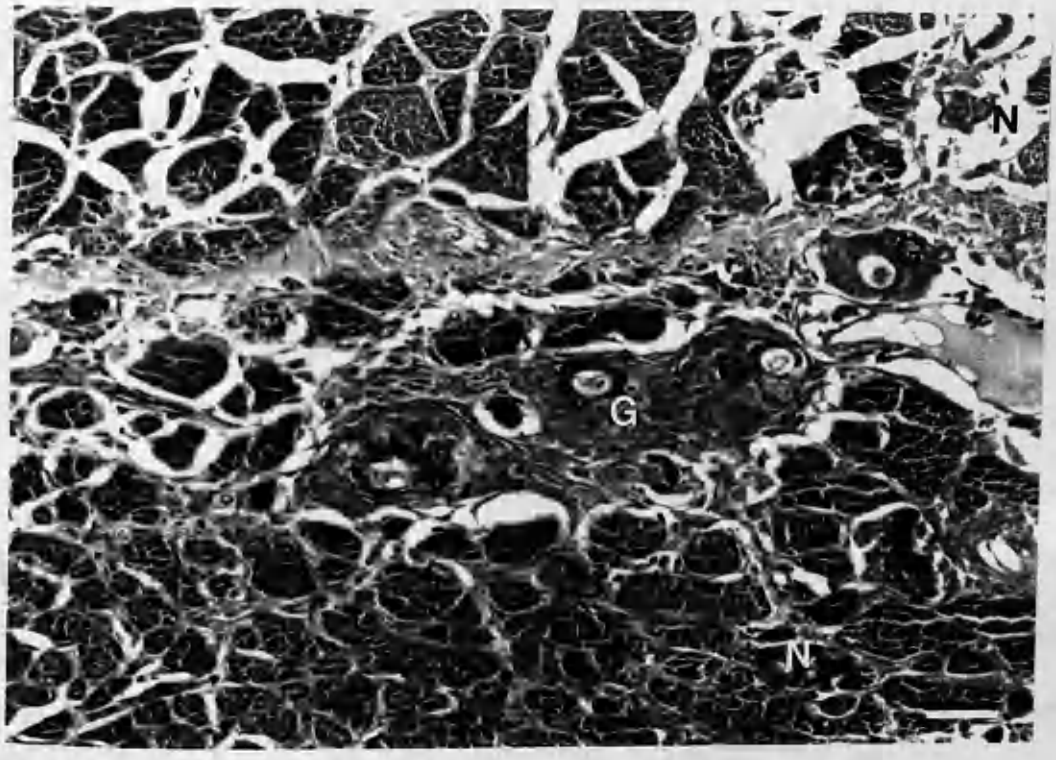
Figure 5.20 Progressive invasion of fungal hyphae (arrows) from dermis to muscle bundle. Scales and epidermis were eroded away, myonecrosis and mycotic granulomas were shown. H&E, Bar = 82 μm

E
S
D

Figure 5.21 Higher magnification of fish skin showed hyperplasia in epidermis layer, melanin deposit (Me), haemorrhage (Ha) and mycotic granuloma (G). Average diameter of fungal hyphae was 14.3 ± 0.4 μm (mean ± SE). S = scale, H&E, Bar = 40 μm

Figure 5.22 Higher magnification of muscle tissue showing focal myonecrosis (N), mycotic granulomas (G) formed by epitheloid cells, no sign of haemorrhage. Average diameter of fungal hyphae was 16.4 ± 0.8 μm (mean ± SE). H&E, Bar = 40 μm


E
S
Ha
Ha
Me


N
G
N

5.5 Discussion

EUS induction was experimentally achieved with a combination of rhabdovirus isolate T9412 and *Aphanomyces invaderis* strain RF-6 inducing EUS lesions in 100% (20/20) of snakehead fish juveniles held at 20°C. The EUS lesions, shallow ulcers with fungal hyphae, were first seen at the i/m injection site then later as multiple EUS lesions on fish bodies. Histology sections confirmed mycotic granulomas in the EUS lesions. The *Aphanomyces* fungus was re-isolated from muscle tissue beneath the EUS lesions. The virus could be re-isolated from 20% of surviving fish. These 2 pathogens did not induce EUS in fish held at 29°C.

Rhabdovirus alone did not induce EUS but caused haemorrhagic lesions in 25% (5/20) of fish after 10-14 days at 20°C. These changes were the same as those observed earlier with i/m-injected fish (Chapter 4, section 4.3.1). At 29°C, the virus caused only petechial haemorrhages on the skin which were less severe compared to the condition at 20°C even though the virus grew well in cell culture at 30°C (Chapter 6). *Aphanomyces* spores alone were able to attach and to invade the fish at the damaged skin site caused by L-15 cell culture medium injection and EUS lesions developed in 35% (7/20) of fish on day 14 at 20°C. However, most affected fish started to recover after day 24. Normally, spores or hyphae could not invade healthy snakehead fish unless the outer skin protection was broken by insertion or injection of the fungus (Chinabut *et al.*, 1995). At 29°C, the waterborne infection of RF-6 spores did not harm the L-15 medium-injected fish. Eventually, the fungus was found to grow better at 31.5°C than at 18-24°C (Roberts *et al.*, 1993). The collective evidence suggests that the protective mechanisms

of snakehead fish might be slowed at low temperature as found by Chinabut *et al.* (1995).

Successful EUS transmission was achieved by co-habitation experiments with EUS-induced fish and water from the associated diseased aquarium. Histological examination confirmed mycotic granulomas in the ulcerative lesions. EUS transmission was first observed between 8-11 days post-co-habitation in all experimental groups. Balasuriya *et al.* (1990) also reported that EUS transmission by co-habitation with natural EUS-diseased fish in Sri Lanka was first seen 7-10 days post-co-habitation. In a similar experiment conducted during the epizootic in December 1991 in the Philippines, disease transmission was first observed on day 9 post-co-habitation and on day 10 after holding the fish in water from the diseased area at 23-24°C (Cruz-Lacierda and Shariff, 1995). This present study found that disease was transmitted to 100% (15/15) of rhabdovirus-treated fish at 20°C, 68% (17/25) of non-treated fish at 20°C and 40% (8/20) of non-treated fish at 28°C by the end of 14 days. The results also suggested that fish pre-treated with rhabdovirus in this transmission study might still carry a low amount of virus or latent virus which might cause greater sensitivity to the disease than non-treated fish and the spread of the disease was better at 20°C than at 28°C. It was also noted that affected fish at 28°C were more capable of recovery than fish at 20°C. The explanation could be that the defence mechanisms and healing processes worked better at the higher temperature (Anderson and Roberts, 1975; Bullock *et al.*, 1978). However, the mechanism of transmission was not clear from this experiment and the amount of virus present in the transmitter fish or released into the water needs to be examined. Recently, the application of immunogold-electron microscopy and

polymerase chain reaction (PCR) diagnostic methods have indicated that fish surviving IHNV infection remain virus carriers for over 12 months (Leong, 1995). The *Aphanomyces* fungus may spread via primary zoospores as zoosporangia and spores balls were found on the ulcer surfaces of affected fish in the EUS induction experiment. However, the fungal hyphae and spores need to be isolated from the ulcer surfaces and tested (no growth at 37°C) in order to establish identity as the *Aphanomyces* pathogen.

Rapid spreading is one of the most distinctive features of EUS. Half the snakehead fish population in 1 pond were affected within 5 days from first seeing EUS-diseased fishes in an epizootic area (Chapter 3). In the laboratory, the occurrence of EUS was slower than in nature. Fungal hyphae were first seen on the shallow ulcers on day 11 post-infection with *Aphanomyces* spores in the induction experiment and on day 8 in the transmission experiment. However, fungal hyphae might invade the muscle tissue much earlier. In the cases of spore-injected or spore-inserted snakehead fish, the fungal hyphae germinated and started to invade the muscle tissue as early as 2-4 days post-injection at 26-31°C (Chinabut *et al.*, 1995) or 3 days post-insertion at 22°C (Roberts *et al.*, 1993).

How natural transmission of EUS occurred was still in question because the *Aphanomyces* fungal hyphae on the ulcer surface were dead and no reproductive structure, zoosporangia or spore balls were found by Roberts *et al.* (1993). However, the authors suggested that zoosporangia and spore balls might be produced only during the early stages of EUS. In the present study, live aseptate fungal hyphae, zoosporangia and spore balls were found on the ulcer surfaces of fish which might have induced skin

irritation. This finding suggests that the clinical signs produced in the laboratory were an early stage of EUS and that free-swimming primary zoospores might have been released from the spore balls, attached and germinated in selected areas of the fish skin infected by rhabdovirus. In an *in situ* replication experiment with another fish rhabdovirus, VHSV was able to attach and multiply in the epidermis of yearling rainbow trout skin then spread deeper into dermis and hypodermis (Yamamoto *et al.*, 1992).

Histopathological changes following EUS induction and transmission in juvenile snakehead fish were clearly dominated by the invasion of fungal hyphae causing hyperplasia of epidermis and dermis, myonecrosis and mycotic granulomas. There were no significant changes in the internal organs. These findings are similar to those reported in naturally EUS-diseased fish by Roberts *et al.* (1986) and Mohan and Shankar (1995) and also those in the experimental injection or insertion studies with *Aphanomyces* fungus described by Roberts *et al.* (1993) and Chinabut *et al.* (1995). An early invasion area of fish skin (Figure 5.19) indicated that the spores might attach to the epidermis, germinate and then invade through the space between scales to the dermis. At this stage the fungal infection might cause skin irritation, as seen during the observation period (Table 5.4), and hyperplasia of epidermis and dermis. Hyperplasia was most likely to have been induced by the *Aphanomyces* fungus as this type of change was not found in rhabdovirus-infected fish. Interestingly, hyperplasia of epidermis was also observed in fish with early EUS disease in Bangladesh (Roberts *et al.*, 1989). The average diameter of fungal hyphae in muscle tissue of EUS-induced and EUS-transmitted fish ranged from 14.3-16.4 μm which was similar to the size of the type strain *Aphanomyces invaderis* in

natural EUS-affected fish reported as 11.7-16.7 μm in diameter by Willoughby *et al.* (1995).

Important evidence relating to the effect of temperature arose from the experiments as follows:

1. Rhabdovirus caused more damage to fish skin at 20°C than 29°C.
2. Rhabdovirus persisted in the fish longer at 20°C than 29°C. Virus isolation at the end of the experiment on day 30 from surviving fish was 20% in fish held at 20°C and 0% in fish held at 29°C.
3. *Aphanomyces invaderis* strain RF-6 caused skin damage in some fish previously injected with L-15 medium at 20°C but there was no sign of fungal infection at 29°C.
4. A combination of both putative pathogens caused very severe EUS lesions at 20°C but no EUS lesions in fish at 29°C.
5. EUS-affected fish could transmit and cause more severe disease at 20°C than at 29°C.
6. Fish with minor EUS lesions had greater ability to recover at 29°C than at 20°C.

The present results indicate that a virus, especially a rhabdovirus, can help the *Aphanomyces* pathogen to induce more severe EUS disease condition in experimental striped snakehead fish than a single infection with the pathogenic fungus. The virus may infect the fish during the cold period of the year and cause some changes in the outer protection that might allow the *Aphanomyces* pathogen to readily attach and invade the body leading to ulcerative lesions, myonecrosis and mycotic granulomas. Aquatic

micro-organisms including bacteria, protozoa, other fungi and fish parasites can cause further damage at the site of the lesions. It would seem possible that moribund fish can produce a mass of aquatic micro-organisms, particularly fish parasites which can easily attack other healthy fish at the affected site or even other fish species, less or insensitive to rhabdovirus infection, causing small open lesions and leading the secondary bacterial and fungal infections. As over 100 fish species have been recorded as affected by EUS in southeast Asia, it is unlikely that the same rhabdovirus and fungal pathogens are responsible for epizootics in different geographical areas. It is important to note that 3 families of viruses, Rhabdoviridae, Birnaviridae and Reoviridae, have been isolated from EUS-diseased fishes (Saitanu *et al.*, 1986; Hedrick *et al.*, 1986: Roberts *et al.*, 1994) and retroviruses were identified in cell lines derived from fishes which were susceptible to EUS (Frerichs *et al.*, 1991). Therefore, further induction experiments are needed to evaluate combinations of different viruses and the *Aphanomyces* pathogen.

Environmental temperature seems to hold the key to the onset and termination of EUS outbreaks. A low temperature may suppress the fish immune system and/or activate both rhabdovirus and *Aphanomyces* fungus pathogens. A high temperature may re-establish fish immunity and/or inactivate both pathogens. In the early years of epizootics of Thailand, Tonguthai (1985) reported that before the start of an outbreak, the temperature decreased rapidly from 30°C to 20°C within a few days. For this reason, EUS may become established for the first time and then spread extensively to new areas during following epizootics. Once EUS was established in an area, it would easily recur when the temperature decreased to its range which need not necessarily be as low as in

the early epizootics. However, rapid changes of temperature are most likely to cause more serious epizootics.

Interestingly, after the completion of these experiments in May 1994, it was learned that EUS induction using both the rhabdovirus and *Aphanomyces* fungus had been first conducted in 1992 by Millar (1994). Fourteen healthy snakehead fish were injected with rhabdovirus strain T9203 isolated from EUS-diseased snakehead fish in Suphanburi province in 1992. The experimental fish were held in water collected from the diseased fish pond during the epizootic. Four days later, the same fish were bathed with *Aphanomyces* fungal hyphae and spores of strain RF-8. The author reported that classic EUS macroscopic and microscopic lesions were found on day 10 post-infection with fungus. The findings of this present study therefore confirms the successful induction of EUS in the laboratory in 1992. Furthermore, it has now also been shown that using tap water in the experimental system achieved the same results as using pond water in the earlier experiment of Millar (1994). Therefore, one possible combination of the complex of etiological agents leading to EUS is a rhabdovirus and an *Aphanomyces* pathogen.

Chapter 6

Characterisation of New

EUS-associated Rhabdovirus Isolates

6.1 Introduction and Literature Review

The name 'Rhabdovirus' first appeared in the classification of viruses in 1966 (Melnick and McCoombs, 1966 cited by Brown, 1987). Brown stated that rhabdovirus was adopted by the International Committee on Nomenclature of Viruses (ICNV) in 1970 and derived from the word 'rhabdos' meaning 'rod-shaped' in Greek. The ICNV became the International Committee on Taxonomy of Viruses (ICTV) in 1973 and over 60 virus families were recognised (Frederick and Kingsbury, 1990). Members of the family Rhabdoviridae caused diseases in animals and plants and were believed to have a more extensive distribution than other virus families (Wagner, 1990). Over 100 rhabdoviruses have been recorded but only 2 genera, *Vesiculovirus* and *Lyssavirus*, were accepted under the family Rhabdoviridae and no generic name has yet been assigned for fish, bird, arthropod, or plant rhabdoviruses (Frederick and Kingsbury, 1990). Later, however, a revised Classification and Nomenclature of Viruses included the fish rhabdoviruses as members of the *Vesiculovirus* and *Lyssavirus* genera according to the number and mobility pattern of the structural proteins (Wunner and Peters, 1991). The following year, a proposed Classification and Nomenclature of Fish Viruses was published by Ahne (1992). The author proposed the use of 'trivial names' for fish rhabdoviruses, based on the 1991 revised classification, such as *Lyssavirus ophicephalus* for SHRV and *Vesiculovirus ophicephalus* for UDRV. At about the same time, fish

rhabdoviruses were grouped as 'lyssavirus-like electrophoreotypes' and 'vesiculovirus-like electrophoreotypes' (Winton, 1992). SHRV was placed in the first group and URDV in the second. Although these fish virus classifications have not yet been generally accepted by fish virologists, the mobility patterns of viral proteins play an important role in all classification schemes.

Fishes represent nearly 50% of species diversity among vertebrate species (Wolf, 1984) and 60 different fish viruses have been found or isolated (Ahne, 1992). The latter author also indicated that rhabdoviruses represented the largest group among the fish viruses which caused disease and losses in economically important culture fishes. The development of world aquaculture has also caused movement of fishes from one area to another which presents a risk of introducing new virus, types or variants into an area. A single disease of fish might be found in many geographic areas caused by the same virus which may, however, comprise different types. Such a wide distribution has been found for a rhabdovirus disease of salmonids, IHN, in which the virus has been isolated from diseased fishes in North America, East Asia and Europe. However, it is recognised that without any fish movement by man, a specific type or variant of this rhabdovirus was usually found only in a limited geographical area (Hsu *et al.*, 1986; Arkush *et al.*, 1989).

In the case of EUS-associated rhabdoviruses, isolates from Asia could be separated into at least 2 groups by protein-gel electrophoresis. One group was similar to *Vesiculovirus* and the other to *Lyssavirus* (Kasornchandra *et al.*, 1992b). A lack of virus isolates from different areas caused difficulty in understanding the relationship between EUS-associated rhabdoviruses and geographic distribution. In the present study, 9 new

viruses were successfully isolated from EUS-diseased fishes in different locations in central Thailand (Chapter 3). Interestingly, all isolates belonged to the family Rhabdoviridae and one of them, used in experimental challenges, appeared to be a fish pathogen and played a significant part in the complex etiology of EUS (Chapter 4-5). Basic physical, biochemical and serological characteristics of these new rhabdovirus isolates need to be compared to provide a greater understanding of the Epizootic Ulcerative Syndrome and also some further data towards understanding the geographic distribution and relationship to other fish rhabdoviruses.

When fish tissue culture was achieved in late 1950's, scientists began to use fish cell lines to isolate viruses from diseased fishes (Wolf, 1988). In the early 1960's, a fish virus isolated from diseased rainbow trout in Europe was named viral haemorrhagic septicaemia virus (VHSV) (Jensen, 1963; Jensen, 1965). VHSV possessed a bullet-shaped morphology that classified the virus as a member of the family Rhabdoviridae and represented the first isolation of a fish rhabdovirus (Wolf, 1988). More fish rhabdoviruses were subsequently recovered from diseased and normal fishes and up to 1979 five additional different fish rhabdoviruses had been isolated (Wolf and Mann, 1980). Recent reports have shown that at least 27 fish rhabdoviruses have now been isolated comprised of 24 isolates between 1963-1986 (Winton, 1992) and 3 further isolates between 1992-1995 (Bovo *et al.*, 1995; Jørgensen *et al.*, 1993; Koski *et al.*, 1992).

Fish rhabdoviruses possess the typical morphological and physico-biochemical characteristics of other vertebrate, invertebrate (including insects) and plant

rhabdoviruses that brought them together in a single family, Rhabdoviridae (Mathews, 1982). Bullet- or bacillus-shaped virus particles measure 150-190 nm in length and 65-75 nm in diameter and possess projecting spikes 10 nm in length on the envelope. The structural components of the virion consist of a helical nucleocapsid core surrounded by a lipoprotein envelope. Rhabdoviruses possess negative sense, single stranded, non-segmented genomic RNA tightly packed inside the virion core. Most virions are assembled at the plasma membrane but a few are found in cytoplasmic vacuoles (Darlington *et al.*, 1972; Wagner, 1987). Sedimentation coefficients of rhabdoviruses were found to be between 38-40S and buoyant densities in sucrose gradients range from 1.14-1.20 g/ml. These viruses are sensitive to lipid solvents, heat at 56°C and low pH. Fish rhabdoviruses infect a broad range of fish cell lines with similar cytopathic effects. The infected cell cultures usually show rounding up of cells followed by cells lysis (Frerichs, 1989). Generally, cold-water fish rhabdoviruses replicate at temperatures ranging from 2-20°C, while warm-water fish rhabdoviruses have higher growth temperatures ranging from 15-30°C (Wolf and Mann, 1980).

Viral protein composition and serological properties have been used for virus characterisation. Analysis of viral proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has become widely accepted as a standard procedure for classifying fish rhabdoviruses as different sizes of viral protein are separated and show specific mobility patterns in the gel. A further serological step following SDS-PAGE is Western blotting in which the separated proteins in the gel are transferred to immobilised membranes (Towbin *et al.*, 1979) and an enzyme-linked immuno assay is applied to determine the antigenic properties of the viral proteins.

A standard nomenclature for the structural proteins of rhabdoviruses has been designated following general agreement at the meeting 'Colloque International sur les Rhabdovirus' in June, 1972 in France (Wagner *et al.*, 1972). The mobility pattern of structural proteins in polyacrylamide gel electrophoresis of vesicular stomatitis virus (VSV) serotype Indiana, prototype *Vesiculovirus*, was used for protein designations. The 5 major proteins of VSV were named as L (large), G (glycoprotein), N (nucleocapsid) , NS (non-structural) and M (matrix) protein with molecular weights approximately 190, 69, 50, 40-45 and 29 kDa. These protein designations also applied to rabies virus, prototype *Lyssavirus*, with some alteration. Rabies virus contained similar L, G and N proteins but no NS protein while the M protein showed 2 bands named M1 and M2 protein. The NS protein was originally regarded as a non-structural protein but it was later agreed that this was a minor structural protein associated with the nucleocapsid core (Wagner *et al.*, 1972). The structure and function of NS and M proteins of *Vesiculovirus* are closely related, respectively, to the M1 and M2 proteins of *Lyssavirus* (Dubois-Dalcq *et al.*, 1984). Protein G is located in the projecting spikes and transmembrane protein but only the spikes are responsible for attachment to the host cell membrane while protein M is responsible for maintaining the strength of virion structure and regulates transcription. Proteins L and NS are associated with a protein N-RNA complex in the virion nucleocapsid core and possesses RNA-dependent RNA polymerase. The replication cycle of rhabdoviruses has been intensively studied in VSV for which the N, NS, M, G, and L proteins are synthesised sequentially as coded in genomic RNA (Wagner, 1990).

Antiserum against the glycoprotein of VSV contained virus-neutralising activity which was not found in antiserum to N protein (Kang and Prevec, 1970; Kelley *et al.*, 1972). The similar property of antiserum against G protein was also reported for rabies virus, and mice vaccinated with purified G protein were protected from subsequent infection with the same virus (Wikter *et al.*, 1973; Cox *et al.*, 1977). More extensive studies on the antigenic properties of VSV indicated that the 5 structural proteins were all antigenic and could be detected by Western blot and Ouchterlony immunodiffusion tests using polyclonal antibody to mono-specific structural protein (Harmon and Summers, 1982).

The structural proteins of fish rhabdoviruses possess similar mobility patterns in PAGE to higher vertebrate rhabdovirus prototype *Vesiculovirus* and *Lyssavirus*. Two salmonid rhabdoviruses, IHNV and VHSV, have the same number of structural proteins and mobility patterns corresponding to *Lyssavirus* while the mobility patterns of 2 non-salmonid rhabdoviruses, SVCV and PFRV, are similar to *Vesiculovirus* (de Kinkelin *et al.*, 1974; Lenoir and de Kinkelin, 1975; McAllister and Wagner, 1975). Labelling the rhabdoviruses with specific radioisotopes such as [^{3}H]glucosamine and [^{32}P]phosphoric acid in a PAGE system demonstrated the types of viral protein present. The G protein of these 4 rhabdoviruses was glycoprotein located at the projecting spikes of virus particles (Lenoir and de Kinkelin, 1975). [^{32}P] was found in association with both N and M protein of IHNV, N protein of VHSV (McAllister and Wagner, 1975) and NS protein in SVCV and PFRV indicating phosphoprotein (Clerx and Horzinek, 1978).

Serum neutralisation tests have clearly identified the serological relationships among fish rhabdoviruses, some of which could be grouped together as the same serotype. Cross-

neutralisation demonstrated that IHNV, VHSV and PFRV were serologically distinct and also differed from SVCV and swim-bladder inflammation (SBI) virus while the latter 2 viruses were serologically identical (Hill *et al.*, 1975). Grass carp rhabdovirus (GRV) isolated by Ahne (1975) and PFRV isolated by de Kinkelin *et al.* (1973) had similar localisations of structural proteins in SDS-PAGE and 4 precipitation lines in immunoelectrophoresis. It was therefore suggested that GRV was a strain of PFRV (Clerx and Horzinek, 1978). Jørgensen and Olesen (1987) demonstrated that a rhabdovirus isolated from cod ulcus syndrome (Jensen *et al.*, 1979) was serologically similar to VHSV. Available information shows that at least 11 serologically different fish rhabdoviruses have been identified (Table 6.1).

Over 70 isolates of IHNV from diseased salmonids from different geographical areas in North-western America were narrowed down to 5 electrophoreotypes (Hsu *et al.*, 1986) but every type shared a common G protein (Engleking *et al.*, 1991). All VHSV isolated from diseased trout in Scandinavia and Italy were believed to comprise 2 separate serotypes in serum neutralisation tests with rabbit antiserum against VHSV strain F1 (Jørgensen, 1972). A more recent report indicated that VHSV from Europe and North America shared some degree of antigenic similarity which allowed classification as a single VHSV group (Winton, 1992). Unlike the above 2 salmonid rhabdoviruses,

Table 6.1 Reported serological comparisons of fish rhabdoviruses using serum neutralisation tests. At least 11 serologically distinct rhabdoviruses have been recorded.

Rhabdovirus	Code	Serological differences	References
1. Viral haemorrhagic septicemia virus	VHSV	IHNV, SVCV, PFRV	Hill *et al.*, 1975
2. Infectious haematopoietic necrosis virus	IHNV	VHSV, SVCV, PFRV	Hill *et al.*, 1975
3. Spring viremia of carp virus	SVCV	VHSV, IHNV, PFRV	Hill *et al.*, 1975
4. Pike fry rhabdovirus	PFRV	VHSV, IHNV, SVCV	Hill *et al.*, 1975
5. *Rhabdovirus anguilla*	EVA, EVX	VHSV, IHNV, SVCV, PFRV	Hill *et al.*, 1980
6. European eel rhabdovirus	B_6 , B_{12} , C_{26}	VHSV, IHNV, SVCV, EVA, EVX, PRV	Castric and Chastel, 1980; Castric *et al.*, 1984
7. Perch rhabdovirus	PRV	VHSV, IHNV, EVA	Dorson *et al.*, 1984
8. Hirame rhabdovirus	HRV	VHSV, IHNV, SVCV, PFRV, EVA, EVX	Kimura *et al.*, 1986
9. Ulcerative disease rhabdovirus	UDRV	VHSV, IHNV, SVCV, PFRV, EVA, EVX, HRV, SHRV	Frerichs *et al.*, 1986; Kasornchandra *et al.*, 1992
10. Snakehead rhabdovirus	SHRV	VHSV, IHNV, SVCV, PFRV, EVX, HRV, UDRV	Ahne *et al.*, 1988; Kasornchandra *et al.*, 1992
11. Carpione rhabdovirus	583	VHSV, IHNV, SVCV, PFRV, EVX	Bovo *et al.*, 1995
European lake trout rhabdovirus †	903/87	VHSV, IHNV, SVCV, PFRV, EVX,	Koski *et al.*, 1992;
European lake trout rhabdovirus *,†	903/87	VHSV, IHNV, SVCV, PFRV, EVX, HRV, Carpione 583, UDRV, SHRV, (PRV, DK 5533)	Björklund *et al.*, 1994
Pike rhabdovirus *,†	DK 5533	VHSV, IHNV, SVCV, PFRV, EVX, HRV, Eel B_{12}, Carpione 583, UDRV, SHRV, (Lake trout 903/87, PRV)	Jørgensen *et al.*, 1993
EUS-associated rhabdovirus ‡	T9204	UDRV	Lilley and Frerichs, 1994

† Variable degrees of serological similarities were reported among European lake trout rhabdovirus (903/87), pike rhabdovirus (DK 5533) and perch rhabdovirus (PRV).

* Serological differences were tested using immunofluorescence technique.

‡ Rhabdovirus isolate T9204 was serologically different to UDRV but the relationship to other rhabdoviruses has not been tested.

Note; Lyssavirus-like eletrophoreotype was reported for rhabdovirus numbers 1, 2, 6, 8, 10, 11, and T9204.
Vesiculovirus-like electrophoreotype was reported for rhabdovirus numbers 3, 4, 5, 7, 9, and 903/87, DK 5533.

the EUS-associated UDRV and SHRV isolates originally recovered from the same disease condition but in different geographical areas, possessed different structural proteins and mobility patterns. Serum neutralisation tests revealed serological dissimilarity between UDRV and SHRV and from other fish rhabdoviruses indicating different serotypes (Ahne *et al.*, 1988; Frerichs *et al.*, 1986; Kasornchandra *et al.*, 1992).

Although viruses belonging to Birnaviridae and Reoviridae families were also isolated from EUS diseased fishes in Asian countries (Frerichs, 1995), virus isolation studies have given a greater frequency of rhabdovirus isolation than other viruses. Surprisingly, 2 mobility patterns of structural proteins and serotypes of rhabdoviruses have been found from the same disease. Therefore, physical and biochemical characterisation and serological comparisons between the new and 2 known earlier UDRV and SHRV isolates of EUS-associated rhabdoviruses were indicated in the present study. Virus neutralisation and Western blot analysis were also applied to assess the immune response of EUS-recovered snakehead fish code AV9420 (section 3.3.4, Chapter 3).

6.2 Objectives:

-To characterise and compare 3 new rhabdoviruses isolated from different EUS-diseased fishes and pond locations.

- To characterise and compare the structural proteins and serological relationships of 3 new rhabdovirus and 5 former rhabdovirus isolates.
- To conduct preliminary observations on the antibody response to EUS rhabdovirus in recovered fish.

6.3 Materials and Methods

Selection of Virus Isolates for Characterisation

Three new virus isolates were selected for characterisation studies. Isolate T9415 (AV9404/10) was originally recovered from internal organ tissue of live EUS-diseased snakehead fish. The second isolate T9416 (AV9404/11) was obtained from pooled muscle and internal organ tissue of live EUS-diseased three-spot gouramis. These diseased fishes were collected from a rice paddy field in the Bangkok area. Isolate T9429 (AV9405/10) was recovered from muscle tissue of live EUS-diseased snakehead fish collected from Suphanburi site 1 (Chapter 3). These 3 new isolates were compared with UDRV strains SL11, BP and 20E, snakehead rhabdovirus SHRV and a relatively recent isolate T9204. Virus isolation details are described in Table 6.2.

6.3.1 Physical and Biochemical Properties of 3 New Rhabdovirus Isolates

6.3.1.1 Virus Morphology

Virus Morphology in Ultra-thin Sections

Isolates T9415, T9416 and T9429 were diluted to 100 $TCID_{50}$/ml in medium L-15 containing 2% FCS and ×1 antibiotics (maintenance medium).

Table 6.2 Rhabdovirus isolates used in comparative structural and serological studies.

Rhabdovirus isolate	Fish host	Location of EUS outbreak	Date of isolation	Reference
T9415	Striped snakehead, *Channa striatus* (Bloch)	Bangkok, central Thailand	January, 1994	Chapter 3
T9416	Three-spot gourami, *Trichogaster trichopterus*	Bangkok, central Thailand	January, 1994	
T9429	Striped snakehead	Suphanburi, central Thailand	January, 1994	
20E	Freshwater eel, *Fluta alba*	Rangoon, Myanmar	December, 1985	Frerichs *et al.*, 1989b; Lilley and Frerichs, 1994
BP	Striped snakehead	Udon Thani, northeast Thailand	October, 1985	
SL11	Mud murrel, *C. punctata* (Bloch)	Colombo, Sri Lanka	February, 1988	
SHRV	Striped snakehead	Nakorn-Pathom, central Thailand	Between 1983-1985	Wattanavijarn *et al.*, 1986; Ahne *et al.*, 1988; Kasornchandra *et al.*, 1991
T9204	Striped snakehead	Suphanburi, central Thailand	January, 1992	Lilley and Frerichs, 1994

One ml of each preparation was inoculated on to preformed monolayers of FHM cells in 25 cm^2 flasks. The viruses were allowed to adsorb to the cells for 1 h at 25°C. Un-adsorbed virus was removed by pipette and the monolayer then washed twice with 5 ml PBS. After the final wash, 5 ml of maintenance medium was added to each flask. Cultures were incubated at 25°C for 40 h. The rhabdovirus-infected FHM cells were processed for transmission electron microscopy as described previously (section 2.3, Chapter 2). Virus particles were photographed and measured.

Virus Morphology in Pelleted Virus Suspensions

Viruses were propagated in 25 cm^2 flasks of SSN-1, RSN-2 or BF-2 cell lines. The RSN-2 cell line was derived from giant snakehead fish, *Channa micropeltes* (Fowler), at

the Institute of Aquaculture. When complete CPE was reached, supernatant fluids were clarified at 3000 rpm (1100×g) at 4°C for 20 min. Virus in the clarified fluid was pelleted at 28500 rpm (100000×g) for 1½ h in a SW41Ti rotor in a Beckman L80 ultracentrifuge. Virus pellets were re-suspended in PBS and re-pelleted as before. The virus pellets obtained were re-suspended in 30-50 µl PBS. EM grids were coated with formvar film according to Hayat (1989) and 10 µl of pellet suspension dropped on the coated grid and allowed to adhere for 1 min. Excess fluid was removed using filter paper and the grid stained with 10 µl phosphotungstic acid. Grids were rinsed with distilled water and air dried then observed under Philips 301 transmission electron microscope at 80 kV.

6.3.1.2 Optimum Growth Temperature

Preparations of rhabdoviruses T9415, T9416 and T9429 containing 3.17, 3.50 and 3.83 $\log_{10}$ $TCID_{50}$/ml in maintenance medium were used. Three 80 cm^2 flasks of 95% confluent FHM passage 135 cells were trypsinised and suspended in 240 ml L-15 medium containing 10% FCS and 1× antibiotics. Eight ml of cell suspension was transferred to 28 flasks of 25 cm^2 flask size. Cell cultures were then incubated at 25°C. After 2 days incubation, the cells had achieved 80% confluency and were ready to inoculate. Twenty-four cell culture flasks were separated into 3 groups of 8 flasks. For the first group of 8 flasks, the culture medium was carefully pipetted off and 1 ml of rhabdovirus T9415 inoculated into each flask and allowed to adsorb for 1 h at 25°C. Unattached rhabdovirus remaining in the inoculum was removed by pipette and the cell monolayer washed twice with 6 ml PBS pH 7.4. Eight ml of maintenance medium was

then added to each flask. With the second and third groups of flasks, the same inoculation procedure was followed using rhabdovirus isolates T9416 and T9429. A control group of 4 flasks was inoculated with maintenance medium instead of virus suspension. Two flasks of each rhabdovirus inoculation and 1 control flask were incubated at 15, 20, 25 and 30°C.

Ten minutes post-incubation, a first set of samples was taken only from rhabdovirus-inoculated flasks incubated at 25°C. 200 µl of supernatant from 2 flasks of each rhabdovirus were pooled and kept in sterile plastic bijou bottles at 4°C. The culture flasks were then returned to the incubator. Further samples were taken daily from every incubation temperature for 7 days. The amount of rhabdovirus in each sample was determined within the next 7 days by end point dilution assay. Assays were performed in duplicate and $TCID_{50}$/ml calculated using the method of Kärber (1931).

6.3.1.3 One-step Growth Cycle

The experiment was carried out using 6-well plates. Three 80 cm^2 flasks with confluent monolayers of FHM passage 136 cells were trypsinised and dispersed to single cell suspensions. Cells were counted and the suspension diluted to 3.28×10^5 cells/ml with culture medium. Three ml of cell suspension was seeded into each well of 16 plates. The plates were covered and sealed with flexible film then incubated at 25°C. Two days later the cells had achieved ~80% confluency and the culture medium was removed. Fifteen plates were inoculated with stock preparations of rhabdovirus isolates T9415, T9416 and T9429 containing 4.17, 4.83 and 4.50 $\log_{10}$ $TCID_{50}$/ml. Each plate received

the 3 virus isolates in duplicate wells at 0.5 ml/well. The sixteenth plate was inoculated with maintenance medium as a control. Culture plates were incubated at 25°C for 1 h to allow virus adsorption and remaining unattached viruses were removed by pipette. Cells of both control and virus infected plates were gently washed twice with PBS at 4-5 ml/well. After the final wash, all 16 plates received 3 ml of maintenance medium per well and were incubated at 25°C. As it was of some concern that unattached virus might still be present in the wells after 2 washes, a first set of virus samples was collected from 1 plate 10 min post-incubation. A series of samples was then obtained at 2, 4, 7, 10, 13, 17, 22, 28, 35, 39.5, 44, 52, 60, and 73 h post-incubation.

At each sampling time, 1 plate was taken from the incubator. Any changes in the FHM cells due to virus infection were observed under an inverted microscope and the development of CPE recorded. Using separate sterile cell scrapers for each virus, the infected FHM cells in duplicate wells were detached from the well surfaces and cells and supernatant fluid pooled and transferred into centrifuge tubes. Each sample of 6 ml was spun at 3000 rpm (1440×g) at 4°C for 20 min in a bench centrifuge. Supernatant fluids were collected and kept in sterile bijou bottles at 4°C. This portion of sample represented ***'free virus'***. The cell pellets remaining in the tubes were subjected to 3 washes with 10 ml PBS with gentle re-suspension and spinning as before to remove free virus. After the final spin, cell pellets for each virus isolate were re-suspended in 1 ml maintenance medium, transferred to separate cryotubes and stored frozen at -20°C. When the final sampling had been completed, all cryotubes were rapidly frozen in liquid nitrogen and thawed in a warm water bath (45°C) for 3 cycles. This portion of sample represented ***'cell-associated virus'***. In order to determine the effect of 3×

freeze-thaw cycles on the viability of the 3 virus isolates, 3 cryotubes containing 1 ml each of virus stock preparation were included in the process.

The amount of virus in each sample was determined by 50% end point dilution assay in FHM cells at 25°C using simultaneous inoculation in 96-well plates. $TCID_{50}$/ml calculation steps were as stated previously (Appendix 1). Each virus isolate generated 32 samples for titration as follows: 15 'free virus' samples; 15 'cell-associated' samples; 1 freeze-thawed stock sample and 1 untreated stock sample. All 96 samples were titrated in duplicate and CPE recorded to day 5 post-infection. Values obtained for cell-associated virus were divided by 6 because the sample volumes were not made up to 6 ml before titration. Moreover, the 3× freeze-thaw cycles were found to cause 0.67, 0.83 and 1.0 $\log_{10}$ $TCID_{50}$/ml reductions in titre of T9415, T9416 and T9429 stocks, respectively. The amounts of cell-associated virus were therefore secondly adjusted by adding the above corresponding value. Values for free and cell-associated virus were combined to give the total amount of virus in the wells. One step growth curves were plotted as $\log_{10}$ $TCID_{50}$/ml against time. One way analysis of variance at 95% confidence level and multiple comparison of all growth studies (including optimum growth temperature) were analysed using Statgraphics software program, STSC, Inc., USA.

6.3.1.4 Stability at Low pH

Tests were conducted in 96-well plates using FHM cells. The 3 rhabdovirus isolates were tested for sensitivity to low pH according to Burleson *et al.* (1992). Virus stocks were propagated in FHM cells in maintenance medium. 100 µl of each isolate was transferred into 900 µl HBSS pH 7.2 as control samples. Another 100 µl was transferred into 900 µl glycine-HCl buffer pH 3.0 (25 ml 0.2 M glycine and 5.7 ml 0.2 N HCl in 100 ml distilled water) as treated samples. Both groups were incubated at 25°C for 1 h. The 50% end point infectivity assays were performed immediately using 10-fold serial dilutions and 4 replicate wells for each dilution. CPE was recorded until day 5 post-infection and $TCID_{50}$/ml calculated and compared. A 1 $\log_{10}$ reduction or more in titre compared to the control indicated sensitivity to low pH.

6.3.1.5 Sensitivity to Heat

The 3 rhabdovirus isolates were tested for sensitivity to heat at 56°C according to Burleson *et al.* (1992). Virus stocks were propagated in FHM cells in maintenance medium and 200 µl of each preparation diluted and mixed well in bottles containing 1800 µl of HBSS. Each diluted virus was equally aliquoted into 3 separate bottles. One bottle of each isolate was held at 4°C as a control and the other 2 bottles were placed in a water bath at 56°C. One bottle of each virus was removed at 30 min and 60 min and transferred to 4°C until titration. The virus preparations held at different temperatures were titrated by 50% end point infectivity using FHM cells in 96-well plates. Ten fold dilutions and 4 replicates for each dilution were used and CPE recorded until day 5.

log_{10} $TCID_{50}$/ml values were calculated and compared. A result of 1 log_{10} reduction or more compared to the control indicated sensitivity to heat at 56°C.

6.3.1.6 Sensitivity to Lipid Solvent

The 3 rhabdovirus isolates were tested for sensitivity to lipid solvent, chloroform, according to Burleson *et al.* (1992). Virus stocks were propagated in FHM cells in maintenance medium. Three groups of the test preparations were set up. For the first group (chloroform-treated viruses), 500 µl of chloroform were added to 1000 µl of each virus in separate sterile glass test tubes. For the second group, 500 µl of chloroform were added to 1000 µl of HBSS in a sterile glass test tube. These groups of tubes were gently shaken for 10 min and then spun at 3000 rpm (1500×g) for 10 min. The upper, transparent liquid phase in each tube was collected and transferred to glass bottles. For the third group, 500 µl of HBSS were added to 1000 µl of each virus in separate bottles as controls. All groups were titrated for 50% end point infectivity using FHM cells in 96-well plates at 25°C. Ten fold dilutions and 4 replicates for each dilution were used. CPE was observed and recorded until day 5 and $TCID_{50}$/ml calculated and compared. A result of 1 log_{10} reduction or more compared to the control indicated sensitivity to lipid solvent.

6.3.1.7 Determination of Nucleic Acid Type

The 3 rhabdovirus isolates were tested for nucleic acid type similar to the method of Rovozzo and Burke (1973). A halogenated pyrimidine, 5-iodo-2-deoxyuridine (IUDR),

was used for the test. Rhabdovirus stocks were propagated in FHM cells. A DNA virus control, channel catfish virus (CCV), was propagated in the BB cell line. Starting with two 96-well plates of confluent monolayers of FHM cells, culture medium was discarded using a multichannel pipette. FHM cells in all wells of the first plate received 100 µl HBSS containing 50 µg/ml IUDR, while the second plate received 100 µl HBSS only. Both plates were used for titrating the 3 rhabdovirus isolates. Another 96-well plate of confluent monolayers of BB cells was used for CCV titrations. Culture medium in all BB wells was first discarded. All wells in the first 6 columns then received 100 µl HBSS containing 50 µg/ml IUDR, while the other 6 columns received 100 µl HBSS only. All 3 plates were incubated at 25°C for 30 min.

The 3 rhabdovirus isolates and CCV were titrated for 50% end point infectivity. Ten fold dilutions in HBSS of all viruses were prepared in separate bottles. Four replicate wells for each dilution of rhabdovirus and 6 replicate wells for each dilution of CCV were applied. Dilutions were added to marked wells and columns at 100 µl/well. Viruses were allowed to incubate in IUDR solution and attach to cells for 2 h at 25°C before supernatant fluids of every well in all plates were discarded using a multichannel pipette. 200 µl of maintenance medium were added each well and the plates were re-incubated at 25°C. CPE were observed and recorded until day 5. $TCID_{50}$/ml values were calculated and compared. A 2 $\log_{10}$ or greater reduction in titre of the IUDR-treated virus compared to the control indicated DNA type viral nucleic acid, while no inactivation effect from IUDR indicated RNA type nucleic acid.

6.3.1.8 Cell Line Susceptibility

The 3 rhabdoviruses were propagated in 25 cm^2 flasks of SSN-1 cells. Culture fluids were harvested after complete lysis of cells and cell debris spun down at 2500 rpm (800×g) for 20 min. Supernatant fluids were collected as virus stocks and held at 4°C. Ten cell lines were selected for susceptibility testing. Five lines, HCK passage 61, HCT passage 31, SSN-1 passage 170, BF-2 passage 121 and EPC passage 137, were derived from warm-water fishes. The other 5 lines, BB passage 133, AS passage 21, FHM passage 135, CHSE-214 passage 249 and RTG-2 passage 131, were derived from cold-water fishes. A simultaneous inoculation procedure with each virus and test cell line was employed. Viruses were titrated for 50% end point infectivity in the 10 cell lines in 96-well plates at 25°C except for CHSE-214 and RTG-2 which were incubated at 20°C. Ten fold dilutions with 4 replicate wells for each dilution were applied. CPE were observed and recorded until day 7. $TCID_{50}$/ml were calculated and compared. A result of any CPE development indicated susceptibility of the cell line to the tested rhabdovirus.

6.3.2 Structural Proteins and Serological Comparisons Between New Isolates and Previous Isolates of EUS-associated Rhabdoviruses

Rabbit Antiserum Preparations

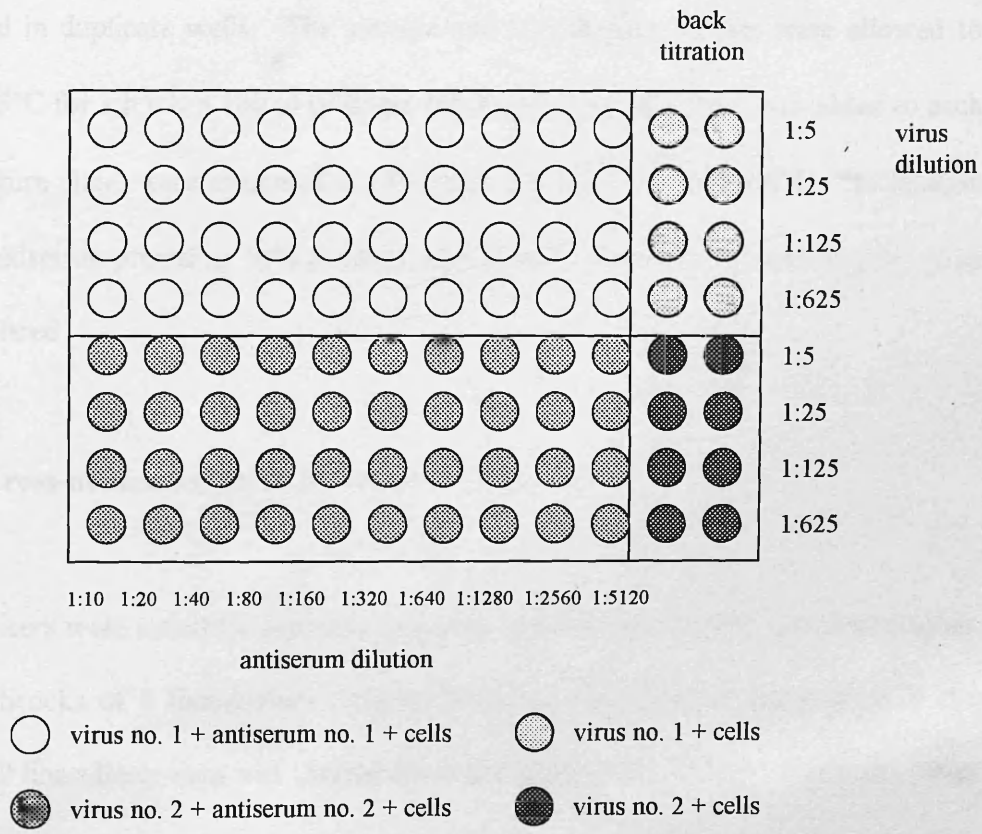
Rabbit antisera against isolates 20E, BP, SL11 and T9204 prepared previously at the Institute of Aquaculture were included in the study. Three new antisera were freshly prepared for this study and methods were similar to the previous 4 antisera. Briefly,

New Zealand white rabbits were used for antisera production. Rhabdovirus isolate T9415 was propagated in RSN-2 cells while isolates T9416 and T9429 were propagated in BF-2 cells. Two 80 cm^2 cell culture flasks were used to grow each virus isolate. Virus was harvested when complete CPE had developed. Cell debris-containing medium was transferred to centrifuge tubes and spun at 3000 rpm (1100×g) at 4°C for 20 min. The clarified fluid containing virus particles was transferred to ultra-clear tubes (Beckman, USA) and virus pelleted by ultracentrifugation at 28500 rpm (100000×g) in a SW41Ti rotor (Beckman, USA) for 1½ h. The supernatant fluid was discarded, the virus pellet re-suspended in 1.5 ml PBS, distributed in 0.5 ml aliquots in 3 bottles and stored at -20°C.

A rabbit immunisation programme was performed according to Hill *et al.* (1981). Equal parts of FCA (Freund's complete adjuvant) and virus suspension were mixed using a micro-emulsifying needle (Stratech Scientific, UK). Each rabbit was inoculated i/m in each hind leg with 0.5 ml of emulsion. Four weeks later, the rabbit received a second injection with a 0.5 ml aqueous aliquot of virus via an ear vein. At week 5, a second i/v inoculum was administered as before. At week 6, the animal was bled out by cardiac puncture under general anaesthetic. Blood was collected in sterile glass bottles and allowed to clot at room temperature for 2-4 h. Serum was recovered, heated at 56°C for 30 min for cytotoxic inactivation (Habashi *et al.*, 1975) and aliquoted into small tubes for storage at -20°C. The clotted blood was kept in a refrigerator overnight and the following day, transferred to centrifuge tubes and spun at 3000 rpm (1100×g) at 4°C for 20 min. The second batch of serum was collected, heated, aliquoted and stored separately at -20°C. Time did not allow the preparation of an anti-SHRV serum.

6.3.2.1 Titration of Neutralising Antisera

Stocks of 7 rhabdovirus isolates were propagated in the SSN-1 cell line and the amounts of virus quantified by titration in SSN-1 cells and expressed as $TCID_{50}$/ml. Each was diluted to ~100 $TCID_{50}$/ml with maintenance medium for use in neutralisation tests. 96-well plates were separated into 2 parts, an upper 4 and a lower 4 rows. Each portion was used for a particular antiserum and its homologous virus. The last 2 columns of the plate were assigned for the back titration of viruses as shown below.

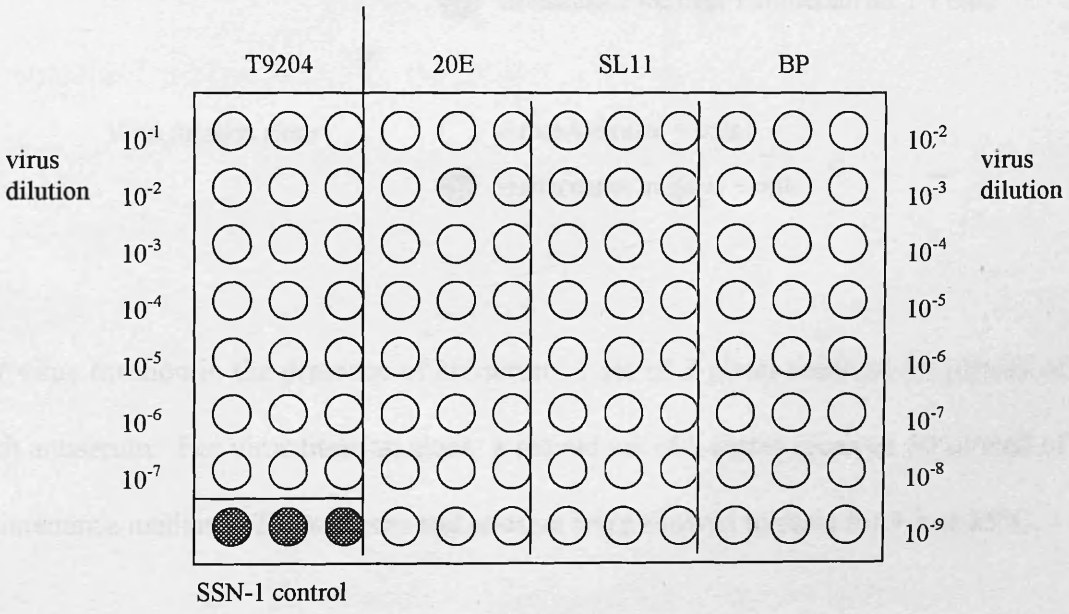


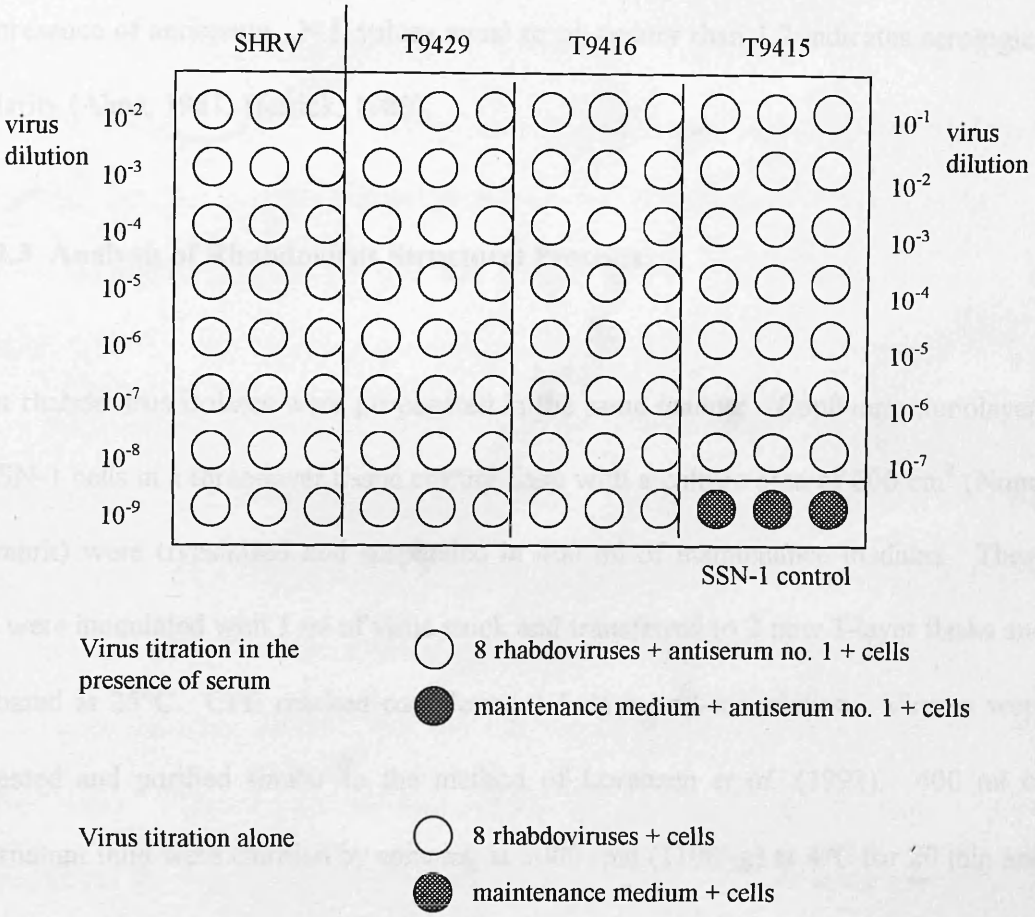
Fifty µl of maintenance medium was added to all wells in the plate except for columns 1, 11 and 12. For each antiserum, 100 µl of antiserum (1:10 dilution) was added to the first column of 4 wells. Serial dilutions 1:2 were prepared by transferring 50 µl from the 4 wells in 1 column to the next column using a 4-channel pipette. Antiserum was mixed well before transferring to the next column. The dilution series was stopped at column 10 and 50 µl from these 4 wells discarded Ten dilutions (1:10, 1:20, 1:40,...,1:5120) of antisera were obtained. Fifty µl of homologous virus was inoculated into each well except for columns 11 and 12. A back titration of the virus was performed using the last 2 columns to confirm the amount of virus used in the system. Viruses were diluted 1:5 in maintenance medium and 4 dilutions, 1:5, 1:25, 1:125 and 1:625, for each rhabdovirus distributed in duplicate wells. The antisera and homologous viruses were allowed to react at 25°C for 1 h when 100 µl of single cell suspensions of SSN-1 was added to each well. Culture plates were incubated at 25°C and CPE recorded until day 5. The dilution of each antiserum providing 50% protection of SSN-1 cells from the homologous virus was calculated.

6.3.2.2 Cross-neutralisation of Rhabdovirus Isolates

Seven antisera were tested for neutralising activity against homologous and heterologous viruses. Stocks of 8 rhabdovirus isolates (including SHRV) were propagated in the SSN-1 cell line. Each virus was titrated alone and against the 7 antisera using 96-well microtitre plate divided into 4 sectors each of 3 columns of 8 rows (3×8 wells). Each sector was used for 1 rhabdovirus isolate. Each row received 1 dilution of virus for 3 replicate titrations. Viruses were prepared as a 10-fold dilution series for 7 or

8 dilutions in an 8-channel box reservoir (ICN, UK). Isolates T9204 and T9415 were limited to 7 instead of 8 dilutions. Row number 8 received maintenance medium for a cell control within the plate. Each virus dilution was transferred to the appropriate wells in the plates using a multichannel pipette. Antisera were diluted with maintenance medium to their appropriate dilutions (1:10 for antiserum T9415, 1:20 for T9416, 1:10 for T9429, 1:40 for T9204, 1:750 for SL11, 1:15 for BP and 1:100 for 20E). Fifteen ml of each antiserum were prepared. Plate layouts are shown below.





For virus titration in the presence of antiserum, 1 set of 2 plates received 50 µl/well of each antiserum. For virus titration alone, a second set of 2 plates received 50 µl/well of maintenance medium. The antigens and antisera were allowed to react for 1 h at 25°C.

Three 80 cm^2 flasks of confluent SSN-1 cells were trypsinised and dispersed to single cell suspensions in 180 ml of maintenance medium. 100 µl of cell suspension were added to each well in the 16 plates and incubated at 25°C. CPE were observed for 5 days. Infectivity ($TCID_{50}$) was calculated and neutralisation indices for each antiserum and the 8 rhabdovirus isolates were compared. The neutralisation index (N.I.) was the

logarithmic difference between $\log_{10}$ $TCID_{50}$ of the virus alone and the virus titration in the presence of antiserum. N.I. values equal to or greater than 1.7 indicates serological similarity (Ahne, 1981; Hetrick, 1989).

6.3.2.3 Analysis of Rhabdovirus Structural Proteins

Eight rhabdovirus isolates were propagated in the same manner. Confluent monolayers of SSN-1 cells in a three-layer tissue culture flask with a culture area of 600 cm^2 (Nunc, Denmark) were trypsinised and suspended in 400 ml of maintenance medium. These cells were inoculated with 1 ml of virus stock and transferred to 2 new 3-layer flasks and incubated at 25°C. CPE reached completion 3-5 days post-inoculation. Viruses were harvested and purified similar to the method of Lorenzen *et al.* (1993). 400 ml of supernatant fluid were clarified by spinning at 3000 rpm (1100×g) at 4°C for 20 min and virus pelleted by spinning at 22500 rpm (67000×g) at 4°C for 2 h in SW 28 rotor in a Beckman L80 ultracentrifuge. Rhabdovirus pellets were re-suspended in 1.5 ml TE buffer (50 mM Tris and 2.5 mM EDTA, pH 7.5). Continuous 15-45% (w/w) sucrose gradients in Ultra-clear tubes (Beckman, USA) were prepared as suggested by Griffith (1986). One ml of each virus pellet suspension was carefully loaded on the top layer of the sucrose gradients. The tubes were placed in a SW28 rotor and spun at 22500 rpm (67000×g) at 4°C for 3 h. One ml of visible virus band was collected from the side of the tubes using syringes and needles. The purified virus harvests were further concentrated by suspending in TE buffer and centrifuging at 28500 rpm (100000×g) at 4°C for 1½ h in a SW41Ti rotor. The purified virus pellets were re-suspended in 100 µl TE buffer, aliquoted and stored at -20°C and -85°C.

Virus structural proteins were analysed by SDS-PAGE using a BRL Mini-V 8.10 system (BRL Life Technologies, UK). Discontinuous SDS-PAGE slab gels were prepared similar to the general procedure described by Hames and Rickwood (1990). Discontinuous buffers, sample buffer and running buffer were prepared according to Laemmli (1970). The resolving gel containing 12% acrylamide was prepared by first mixing 2.5 ml of 1.5 M Tris-HCl pH 8.8, 4.35 ml distilled water, 100 µl of 10% SDS and 3.0 ml of 40% acrylamide/bis (BioRad, USA). The mixture was degassed for 5 min using a vacuum pump before adding 50 µl of 10% ammonium persulphate and 5 µl of TEMED (N, N, N', N'-tetramethylethylenediamine) (Sigma, UK) and loading into 2 sets of glass assembly plates. The gel was overlaid with 200 µl butanol-saturated distilled water. When the gel had polymerised, the overlay was poured off. The top surface of the gel was rinsed with distilled water and dried. The stacking gel contained 4% acrylamide and was prepared by mixing 2.5 ml 0.5 M Tris-HCl pH 6.8, 6.4 ml distilled water, 100 µl 10% SDS and 0.97 ml 40% acrylamide/bis. The mixture was degassed and the same amounts of 10% ammonium persulphate and TEMED were added before loading on the top of the resolving gel. A comb was inserted to make 10 wells in the stacking gel. Polymerisation occurred in 15-30 min.

Sample buffer was a mixture of 1 ml 0.5 M Tris-HCl pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-β-mercaptoethanol and 0.2 ml 0.05% bromophenol blue. Each purified rhabdovirus and low molecular weight markers (BioRad, USA) were mixed separately with an equal volume of the sample buffer which the final concentration contained 0.0625 M Tris-HCl, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol and

0.001% bromophenol blue (Laemmli, 1970). Final dilution of the markers was 1:1280. They were heated in boiling water for 90 sec then rapidly cooled in iced water. Ten µl each of the samples and markers were loaded into separate wells using a Hamilton syringe (Hamilton company, USA).

Proteins of rhabdovirus isolates were separated in the BRL Mini-V system. The running buffer contained 0.025 M Tris base pH 8.3, 0.192 M glycine and 0.1% SDS. A power supply (Gibco BRL model 400L, Life Technologies, UK) was run at 200 V and 200 mA for 45-50 min. Proteins in the gel were fixed in ethanol-acetic acid and stained using a silver stain kit (Sigma, UK) according to the protocol sheet supplied with the kit. Standard molecular weights were plotted on a semilogarithmic scale against their migration distances. Viral protein molecular weights were determined according to Hames and Rickwood (1990).

6.3.2.4 Western Blot Analysis of Rhabdovirus Isolates Using Rabbit Antisera

Eight rhabdovirus isolates were electrophoresed by SDS-PAGE as described in section 6.3.2.3. At the end of a 45-50 min run, gels were carefully removed from the glass plates and transferred to a bath containing 300 ml of pre-chilled protein transfer buffer. The transferring buffer was prepared according to Towbin *et al.* (1979) and contained 24.8 mM Tris base, 192 mM glycine and 10% methanol at pH 8.3. The gels were held in the buffer for 15 min and then assembled in BRL Mini-V system Western blotting apparatus. Viral proteins in the gels were electrically transferred to nitro-cellulose membranes in the pre-chilled protein transfer buffer. Voltage and current were set at

150 V and 130 mA for 1 h as suggested by the manufacturer. After transfer, the nitro-cellulose membranes were rinsed in Tris buffer saline (TBS; 20 mM Tris base, 500 mM NaCl, pH 7.5). Molecular weight marker lanes were cut and stained for 1 h with amido black (0.1% dye in a mixed solution of 45% methanol and 10% acetic acid) and de-stained with a solution of 90% methanol and 2 % acetic acid (Towbin *et al.*, 1979). The remaining part of the membrane which contained immobilised viral proteins was subjected to enzyme-linked immuno-staining in a glass bath at room temperature. Non-specific binding sites on nitro-cellulose membrane were blocked with 10 ml of 1% milk power in TBS for 1 h and the membranes then rinsed 3 times with TBS. Rabbit antisera against isolates T9415, T9416, T9429, T9204 were diluted 1:50 in TBS containing 1.5% bovine serum albumin (BSA) while antiserum 20E was similarly diluted 1:100. Ten ml of antiserum was poured on to the membrane and allowed to bind to specific antigens for 1 h. The un-bound antiserum was removed by washing 5 times for 30 min in TBS containing 0.05% Tween (TTBS). A commercial antiserum against rabbit immunoglobulin G conjugated with horseradish peroxidase enzyme (anti-rabbit-HRP) was diluted 1:1000 in TBS containing 1.5% BSA and 10% FCS. Ten ml of the conjugate was poured on to the membrane and incubated for 1 h. The membrane was rinsed 5 times for 30 min in TTBS. The substrate 3, 3'-dianimobenzidine (SIGMA *FAST* DAB; Sigma, UK) was prepared as recommended by the supplier. The membrane was incubated with 7 ml of the substrate solution for 20-30 min. Brown precipitate bands appeared on the membrane. The membrane was washed in distilled water and air dried.

6.3.2.5 Virus Neutralisation and Western Blot Analysis With Fish Sera: Preliminary Observations.

Virus neutralisation tests were carried out on sera from 16 EUS-recovered snakehead fish (Chapter 3) and 1 normal snakehead fish. Serum samples were diluted 1/5 in maintenance medium and filter-sterilised using 0.45 μm filter-attached syringes. Sera were further diluted 1:2 in 96-well plates using a multichannel pipette. Two replicate wells were assigned for each test serum which were serially diluted 2-fold from 1/10, 1/20,...,1/1280. The same amounts (50 μl) of virus isolate T9429 were added to the wells and allowed to react with the fish sera for 1 h before the SSN-1 cells were added. CPE were recorded until day 5.

A repeat sucrose gradient purified preparation of rhabdovirus T9429 was obtained as previously but using smaller tubes and SW41Ti rotor. Three hundred and fifty μl of pelleted virus suspensions were loaded on top of continuous 15-45% sucrose gradients and spun at 20000 rpm (50000×g) for 3 h. The virus band was collected and again pelleted. The purified virus pellets were re-suspended in 120 μl of TE buffer. A discontinuous SDS-PAGE gel was prepared with 12% and 4% acrylamide resolving and stacking gels. Measured from the top of the resolving gel, the stacking gel was 0.7-0.8 cm high. A comb was not used in this preparation and the whole top length of the stacking gel was used as a loading well. An equal volume of purified isolate T9429 and sample buffer was mixed and heated in boiling water for 2 min. 220 μl of the mixture was loaded on to the top of stacking gel. Two gels were prepared. The first gel was

loaded with the purified T9429 obtained earlier and the second gel was loaded with above preparation.

Proteins of rhabdovirus isolate T9429 were separated and transferred to a nitro-cellulose membrane under the same conditions as described previously. An enzyme immuno staining assay was performed similar to Ristow *et al.* (1993) and Towbin *et al.* (1979). When protein transfer had been completed, the 2 membranes were rinsed in TBS. One was blocked with 1% milk powder in TBS and the other with 3% gelatine in TBS for 1 h. They were then rinsed 3 times in TBS and divided to 20 separate lanes using a multiscreen apparatus (BioRad, USA). Eighteen lanes were used for the test. Lane 18 of the first membrane and lanes 1 and 18 of the second membrane were treated with 600 µl each of 1:100 dilution of rabbit antiserum T9429 in TBS containing 1.5% BSA. These lanes acted as controls and indicators of the location of viral protein bands on the membrane. For the first membrane, lanes 1-17 were treated with 600 µl each of 1:10 dilutions of the sera from 16 EUS-recovered snakehead fish and 1 normal snakehead fish in TBS containing 1.5% BSA and then incubated for 1 h at room temperature. For the second membrane, lanes 2-17 were treated with 600 µl each of 1:40 dilutions of sera from 15 EUS-recovered snakehead fish and 1 normal snakehead fish in TBS containing 1.5% BSA and 10% FCS and incubated overnight at 20°C. After incubation, each lane was washed 5 times with TTBS for 30 min. The second antibody, rabbit antiserum against snakehead fish immunoglobulin (Ig) obtained from AAHRI for which the fish Ig was purified using affinity chromatography according to Smith (1992). It was diluted 1:50 (for the first membrane) and 1:100 (for the second membrane) in TBS containing 1.5% BSA and 10% FCS. All lanes were treated with 600 µl of the second antibody and

incubated at room temperature for 1 h. They were then washed 5 times with TTBS for 30 min. Anti-rabbit-HRP was diluted as stated earlier and 600 μl added to each lane. After 1 h incubation, the membranes were washed 5 times with TTBS then removed from the multiscreen apparatus and placed in glass baths. The substrate solution was added and incubated for 20-30 min. Brown precipitate bands appeared on the membranes. They were washed in distilled water and air dried.

6.4 Results

6.4.1 Physical and Biochemical Properties of New Rhabdovirus Isolates

6.4.1.1 Virus Morphology

Transmission electron micrographs of FHM cells infected virus isolates T9415, T9416 and T9429 revealed similar bacillus- or bullet-shaped rhabdoviruses (Figure 6.1a-c). Rhabdovirus particles were assembled in the cell cytoplasm and also at the cell membrane. The dimensions of 25 virus particles are recorded in Table 6.3.

Table 6.3 Dimensions of new rhabdovirus isolates in ultra-thin sections and virus pellet suspension preparations.

Virus preparation	Dimensions of 6 rhabdovirus isolates (length ± SD nm × diameter ± SD nm)					
	T9415 (AV9404/10) *	T9416 (AV9404/11)	T9429 (AV9405/10)	T9424 (AV9405/5)	T9428 (AV9405/9)	T9430 (AV9405/11)
Virus in ultra-thin sections	177 ± 13 × 53 ± 2 n = 25	175 ± 14 × 48 ± 4 n = 25	174 ± 18 × 50 ± 4 n = 25	nd	nd	nd
Virus pellet suspension	185 ± 16 × 62 ± 7 n = 10	186 × 70 n = 1	215 ± 22 × 71 ± 5 n = 6	180 ± 6 × 58 ± 1 n = 5	193 ± 18 × 65 ± 6 n = 6	209 ± 16 × 63 ± 2 n = 2

* Virus codes in parenthesis refer to code and sample numbers in Table 3.2 and 3.3 in Chapter 3.
nd = not done. n = number of particles counted.

Figure 6.1 Electron micrographs of 3 new isolates of EUS-associated rhabdovirus in ultra-thin sections. All isolates were propagated in FHM cells and exhibited bullet shaped morphology. Bar = 153 nm

a. Rhabdovirus isolate T9415 (177 ± 13 × 53 ± 2 nm).

b. Rhabdovirus isolate T9416 (175 ± 14 × 48 ± 4 nm).

c. Rhabdovirus isolate T9429 (174 ± 18 × 50 ± 4 nm).

A few particles of isolate T9416 were found to be unusually long (over 300 nm). Protruding virus spikes were not readily seen in these preparations but central axial channels observed in cross-sections of the virus particles measured approximately 7-12 nm. Phosphotungstic acid stained pellet suspensions of 9 virus isolates showed similar bacillus- or bullet-shaped rhabdovirus particles. As it had been difficult to obtain good pre-coated EM grids and good virus pellet preparations, only 6 isolates could be photographed and measured (Figure 6.2a-f). A few preparations showed well defined projecting spikes but in most instances it was difficult to determine the length of the spikes and the recorded sizes of all isolates therefore include the length of projecting spikes, if present (Table 6.3). The lengths of projecting spikes were found between 7.5-11.6 nm and about 35 neucleocapsid coils were counted (Figure 6.3). A single rhabdovirus-like particle measuring ~209 × 81 nm was found in the muscle tissue of the EUS-diseased three-spot gourami (Figure 6.4) from which T9416 was isolated.

6.4.1.2 Optimum Growth Temperature

The growth of the 3 rhabdovirus isolates in FHM cells and development of CPE at different temperatures is shown in Table 6.4 and Figure 6.5. After 1 h virus adsorption followed by 2× PBS washes of the monolayer, the amount of virus present in the supernatant fluid after 10 min incubation was found to be less than in the original inoculum with values of 2.0, 2.0 and 3.0 $\log_{10}$ $TCID_{50}$/ml, respectively. Areas of 25% - 50% CPE developed on day 1 and complete CPE was found on day 3-5 in infected cultures incubated at 25-30°C. Changes in infected FHM cells were slow at 15-20°C

Figure 6.2 Electron micrographs of 6 new EUS-associated rhabdovirus isolates in pellet suspensions with phosphotungstic acid staining. Bar = 116 nm.

a. Rhabdovirus isolate T9415 propagated in RSN-2 cell line.

b. Rhabdovirus isolate T9416 propagated in BF-2 cell line.

c. Rhabdovirus isolate T9429 propagated in BF-2 cell line.

d. Rhabdovirus isolate T9424 propagated in BF-2 cell line.

e. Rhabdovirus isolate T9428 propagated in BF-2 cell line.

f. Rhabdovirus isolate T9430 propagated in BF-2 cell line.

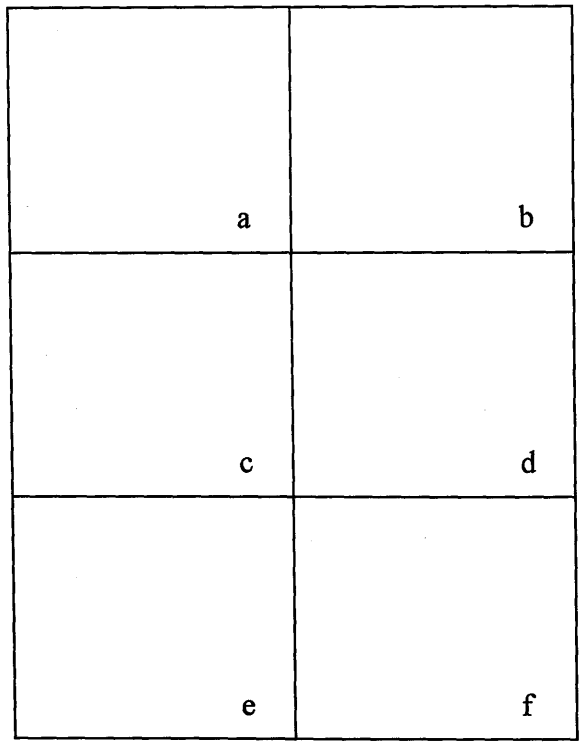
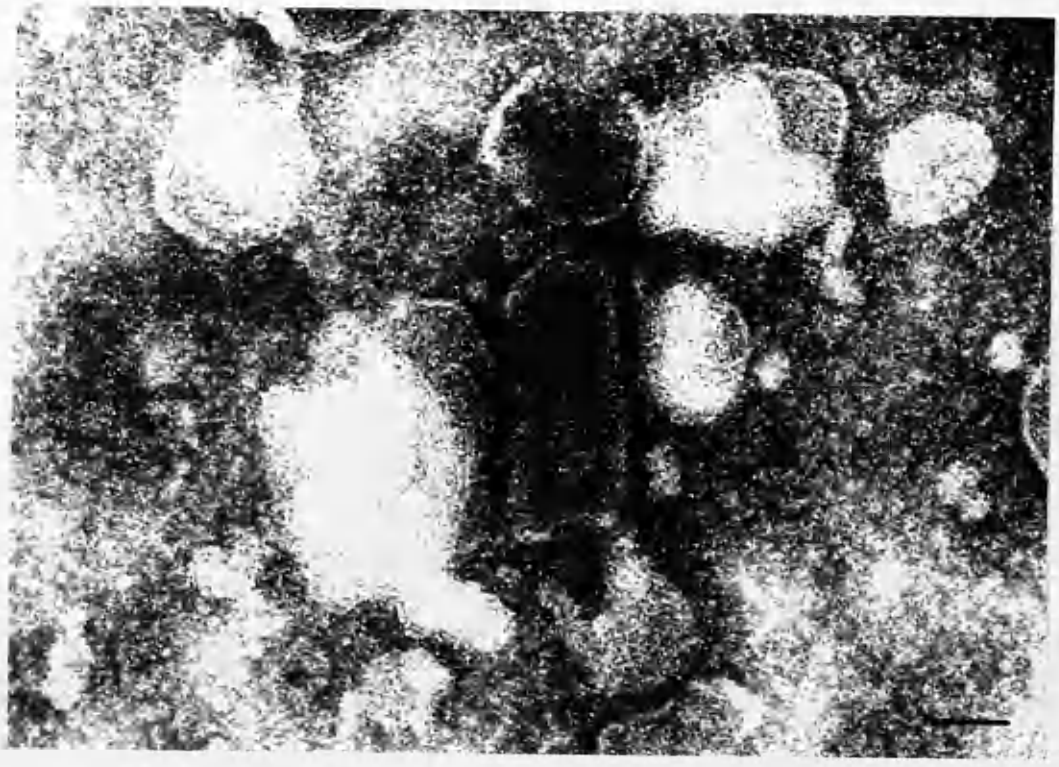


Figure 6.3 Rhabdovirus isolate T9416 showing ~35 coils of neucleocapsid structure ~55 nm in diameter, 165 nm in length and 7.5-8.0 nm projecting spikes.

Bar = 41 nm.

Figure 6.4 Rhabdovirus-like particle (~209 × 81 nm) in the muscle tissue of EUS-diseased three-spot gourami from which rhabdovirus T9416 was isolated.

Bar = 90 nm.



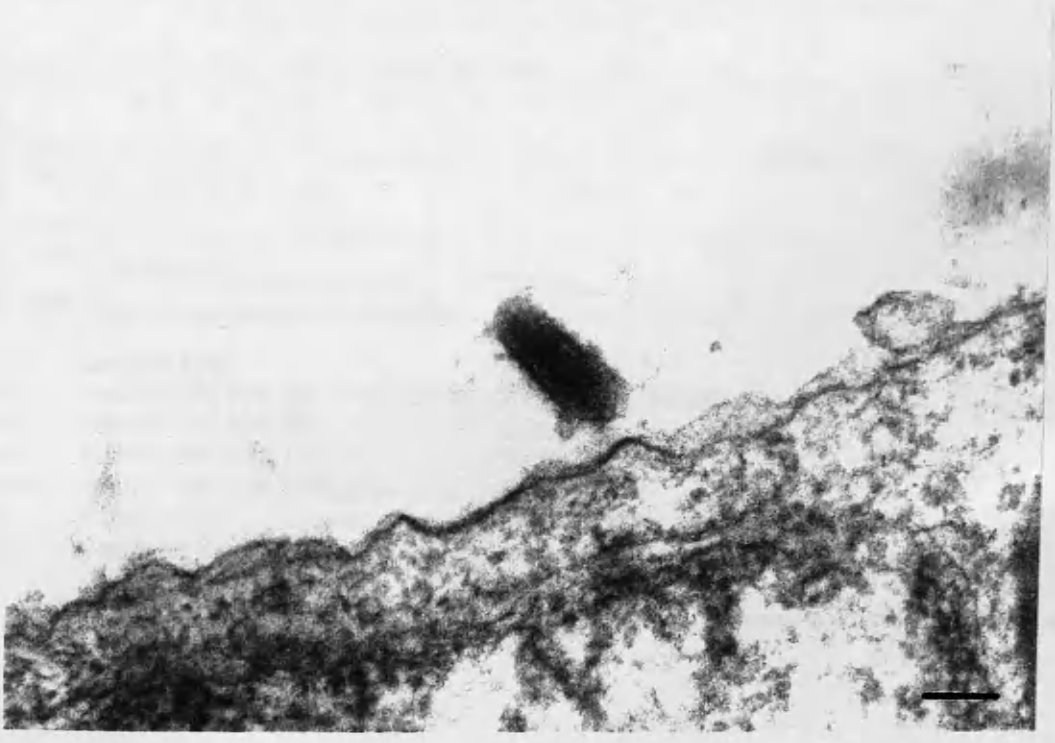


Table 6.4 Development of CPE and growth of 3 rhabdovirus isolates in FHM cells with progressive infection. Preformed monolayers in 25 cm^2 flasks were inoculated with 3.17, 3.50 and 3.83 log_{10} $TCID_{50}$/ml of isolates T9415, T9416 and T9429, respectively. Cultures were incubated at 4 different temperatures and observed for 7 days. Values are presented as log_{10} $TCID_{50}$/ml of virus. One way analysis of variance was observed at 95% confidence level.

Time	T9415 at different temperatures (°C)				T9416 at different temperatures (°C)				T9429 at different temperatures (°C)			
	15	20	25	30	15	20	25	30	15	20	25	30
10 min	- 2.0	- 2.0	- 2.0	- 2.0	- 2.0	- 2.0	- 2.0	- 2.0	- 3.0	- 3.0	- 3.0	- 3.0
1 day	- 3.5	- 4.5	+ 7.0	+ 7.5	- 2.5	- 4.5	+ 6.5	++ 7.5	- 3.0	- 5.0	+ 6.0	++ 6.5
2 days	- 4.0	+ 6.5	++ 8.5	+++ 8.5	- 4.5	+ 6.0	++ 8.0	+++ 7.5	- 4.5	++ 6.5	++ 6.5	+++ 7.5
3 days	- 5.0	++ 6.0	++ 8.0	++++ 8.5	- 5.0	++ 5.5	+++ 8.0	+++ 8.5	+ 5.5	++ 7.0	+++ 7.5	+++ 8.0
4 days	+ 5.0	++ 6.5	+++ 7.5	++++ 8.5	+ 5.5	++ 6.5	+++ 8.5	++++ 8.0	+ 6.0	+++ 7.5	+++ 7.5	++++ 8.0
5 days	+ 5.5	++ 6.5	++++ 7.5	++++ 7.5	+ 5.0	++ 6.5	++++ 8.5	++++ 8.0	+ 7.0	+++ 8.0	++++ 7.0	++++ 8.0
6 days	+ 6.0	++ 7.5	++++ 8.5	++++ 8.5	+ 5.5	++ 6.5	++++ 8.0	++++ 8.5	+ 7.5	+++ 7.5	++++ 7.5	++++ 8.5
7 days	+ 5.5	++ 6.5	++++ 8.0	++++ 7.5	+ 5.5	++ 6.0	++++ 7.5	++++ 8.0	++ 8.5	++++ 7.5	++++ 7.5	++++ 8.0
Sig. level	* $p = 0.02$				$p = 0.01$				$p = 0.39$			
Mean com.	†											

- Negative CPE.
+ Positive CPE with foci of rounded-up cells occupying 10-25% of monolayers.
++ Positive CPE with 50% of monolayers affected.
+++ Positive CPE with 75% of monolayers affected.
++++ Positive CPE with 100% of monolayers affected.
* P value < 0.05 indicates significantly different growth of viruses among 4 incubations.
† Connected lines across column indicates insufficient evidence to verify the differences of growth between 2 means.
Growth comparisons at 15-30°C of different isolates were found non-significant differences at $p = 0.29$, 0.43, 0.81 and 0.98, respectively.

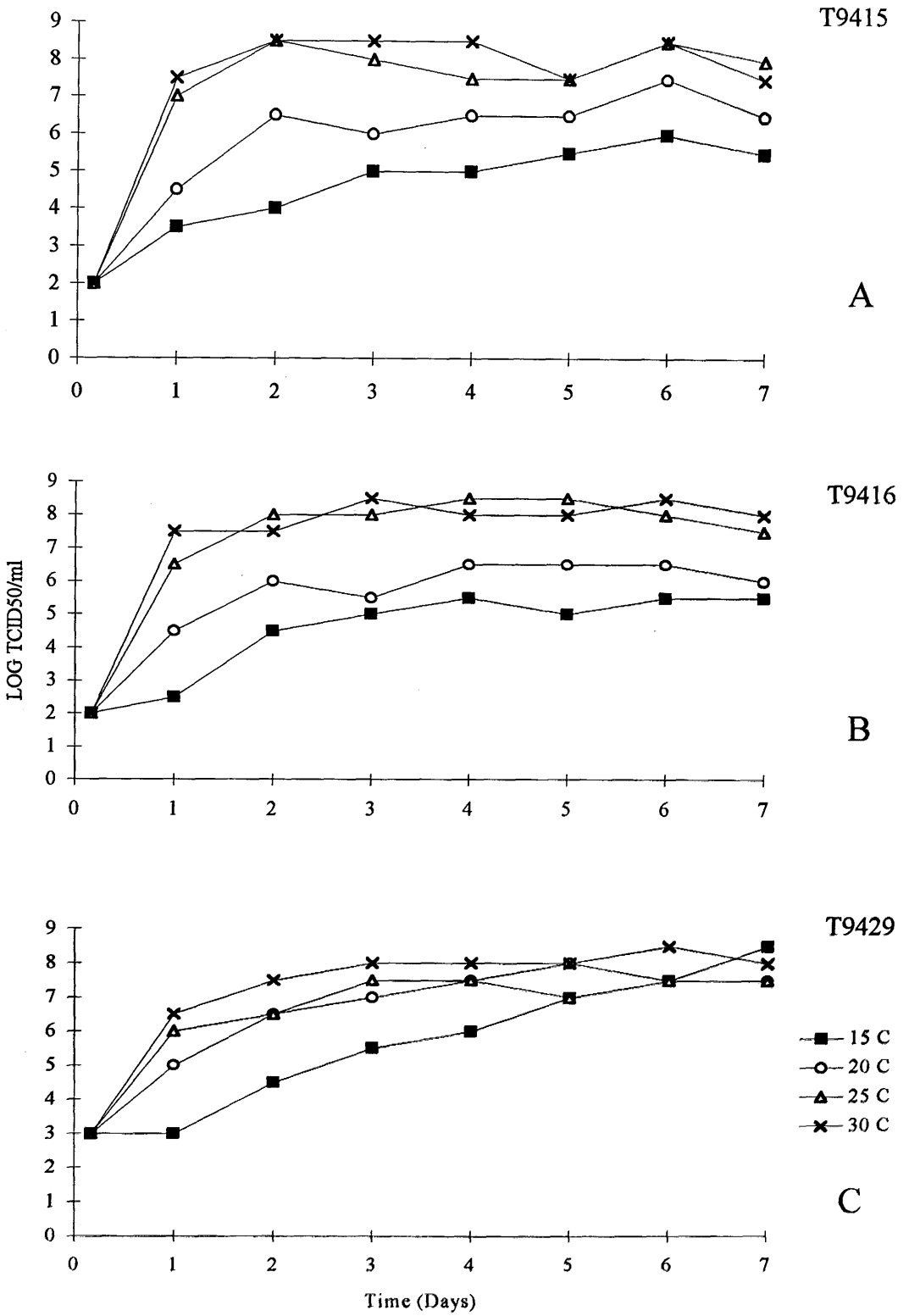


Figure 6.5 Growth of EUS-associated rhabdoviruses in FHM cells at different temperatures. Cells were inoculated with 3.17, 3.50 and 3.83 $\log_{10}$ $TCID_{50}$/ml of isolates T9415, T9416 and T9429. After 1 h incubation and 2× PBS washes, supernatant fluids were collected at intervals and titrated for amount of virus. (A) isolate T9415. (B) isolate T9416. (C) isolate T9429.

and complete CPE did not develop during 7 days observation except for isolate T9429-infected FHM at 20°C. The growth of the 3 viruses increased rapidly within 24 h at higher incubation temperatures. Growth of isolates T9415 and T9416 showed significant differences between the 4 incubation temperatures with p values equal to 0.02 and 0.01, respectively. Growth differences at 15°C and 20°C were non-significant. Growth at 20°C, 25° and 30°C was also not significantly different. Both virus isolates grew significantly less at 15°C than at the 3 other incubation temperatures. Growth of isolate T9429 showed non-significant differences (p = 0.39) between the 4 different incubation temperatures. During the first few days of growth, T9429 was similarly slow to the other isolates but the rate increased after day 2 and was maintained at the same level as the higher incubation temperatures. Isolate T9429 adapted well to the lower temperature of 15°C. Although only 50% CPE was found in T9429-infected FHM on day 7, the released virus in the supernatant fluid had already reached maximum. All isolates grew best at 20-30°C with optimum temperatures of 25-30°C.

6.4.1.3 One-step Growth Cycle

After 1 h incubation and 2 washes of the infected monolayers, samples were collected at intervals over 73 h. Amounts of free virus, cell-associated virus and total virus are shown as $TCID_{50}$/ml (Table 6.5). One-step growth curves were plotted as shown in Figure 6.6. Cytopathic changes during virus infection were noted:

Table 6.5 One step growth cycle of rhabdovirus isolates in FHM cells. Cells were inoculated with 4.17, 4.83 and 4.50 log_{10} $TCID_{50}$/ml of isolates T9415, T9416 and T9429, respectively. After 1 h incubation and 2× PBS washes, maintenance medium was replaced and cultures incubated at 25°C. The viruses were harvested at different periods of time and titres determined as log_{10} $TCID_{50}$/ml.

Time	T9415 (log_{10} $TCID_{50}$/ml)			T9416 ($log_{10}TCID_{50}$/ml)			T9429 (log_{10} $TCID_{50}$/ml)		
(h)	Free virus	Cell-ass virus	Total* virus	Free virus	Cell-ass virus	Total* virus	Free virus	Cell-ass virus	Total* virus
10 min	2.00	1.59	2.14	2.50	1.25	2.52	2.00	1.92	2.26
2	2.00	3.09	3.12	2.50	3.25	3.32	2.00	3.42	3.44
4	4.50	4.09	4.64	5.00	4.75	5.19	4.00	4.92	4.97
7	5.00	6.09	6.12	6.00	5.75	6.19	5.00	6.92	6.93
10	5.50	7.09	7.10	6.00	7.75	7/76	5.50	6.92	6.94
13	7.50	7.59	7.85	7.00	7.75	7.82	6.50	7.92	7.94
17	8.50	8.09	8.64	8.50	8.25	8.69	7.50	7.92	8.06
22	8.50	8.09	8.64	8.50	8.25	8.69	8.50	7.92	8.60
28	8.50	7.59	8.55	8.50	7.75	8.57	8.00	7.92	8.26
35	8.50	7.59	8.55	8.00	7.75	8.19	8.50	7.92	8.60
39.5	8.50	7.59	8.55	8.50	8.25	8.69	8.50	7.92	8.60
44	8.50	7.59	8.55	8.00	8.25	8.44	8.50	7.92	8.60
52	8.00	8.09	8.35	8.00	8.25	8.44	7.50	8.42	8.47
60	8.50	8.09	8.64	8.00	8.25	8.44	7.50	8.42	8.47
73	7.50	7.09	7.64	8.00	7.75	8.19	8.00	6.92	8.04

* Total virus = Log_{10} (anti-log_{10} free virus + anti-log_{10} cell-associated virus).
There was no significant difference between amounts of free virus T9415, T9416 and T9429 (p=0.88).
There was no significant difference between amounts of cell-associated virus T9415, T9416 and T9429 (p=0.39).

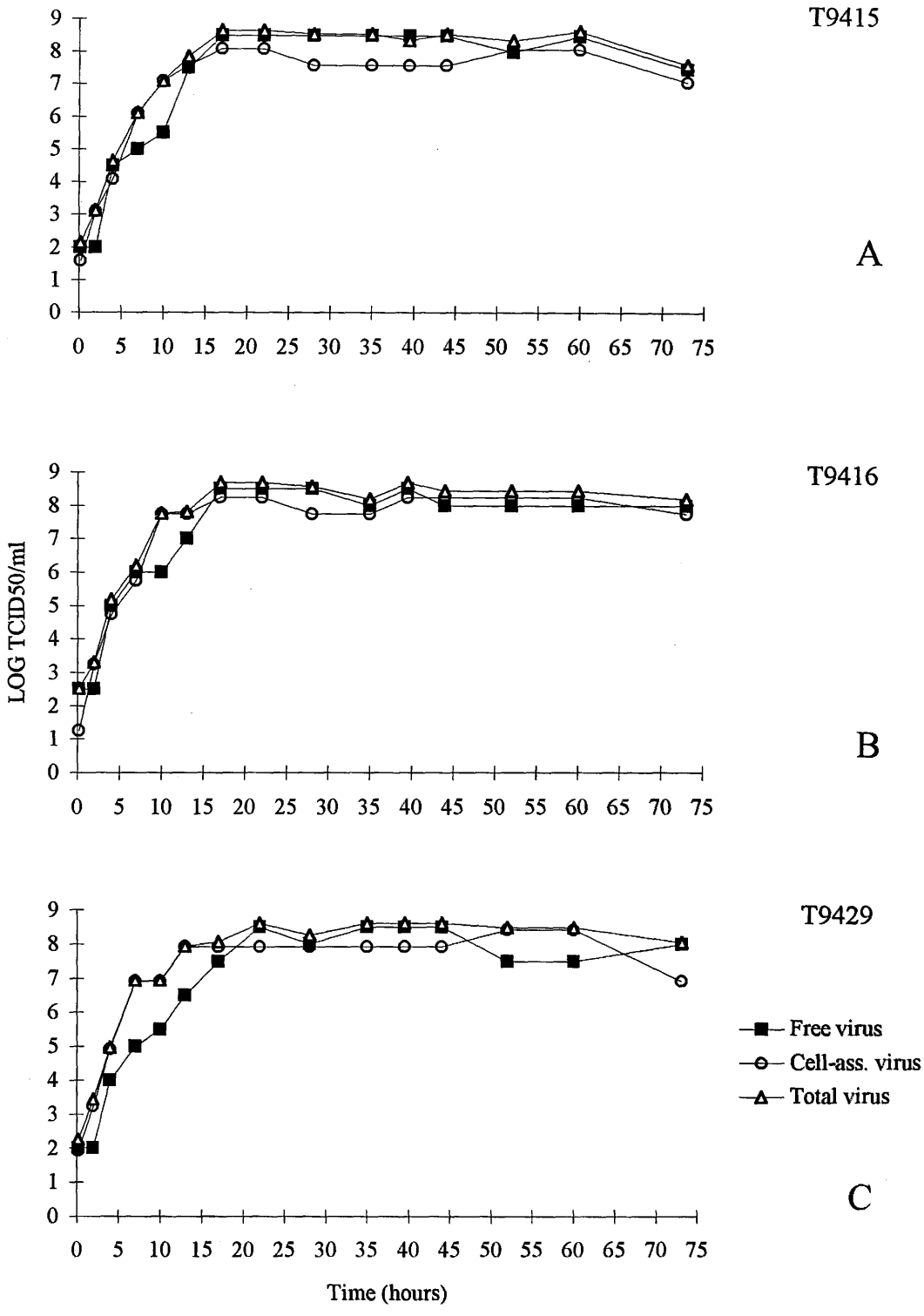


Figure 6.6 One step growth cycle of rhabdovirus isolates in FHM cell line at 25°C. Cells were infected with 4-5 $\log_{10}$ $TCID_{50}$/ml of virus, incubated for 1 h and washed twice with PBS before medium L-15 (2% FCS) was added to cell cultures. (A) isolate T9415. (B) isolate T9416. (C) isolate T9429.

At 10 min, there were no significant monolayer changes except for shrinking and thickening of the edge of FHM cell sheets caused by the movement of medium and PBS washes. Low levels of free virus of isolates T9415, T9416 and T9429 present after PBS washes were assayed at 2.0, 2.50 and 2.0 $\log_{10}$ $TCID_{50}$/ml while cell-associated virus was found at 1.59, 1.25 and 1.92 $\log_{10}$ $TCID_{50}$/ml, respectively.

- At 2 h, monolayer shrinking and thickened edges were still observed. Amounts of cell-associated virus had increased rapidly from 1.25-1.92 to 3.09-3.42 $\log_{10}$ $TCID_{50}$/ml, while free virus levels seemed to have stabilised. New progeny may not have been produced indicating a latent phase of growth of these isolates.
- At 4 h, monolayer shrinking and edge thickening had disappeared and the cell sheet had returned to normal appearance on the plate surfaces but some free, swollen, cells were suspended in the medium. The amounts of free virus had rapidly increased from 2.0-2.50 $\log_{10}$ to 4.0-5.0 $\log_{10}$ $TCID_{50}$/ml. New progeny had started to be released from host cells, while host cells also carried high amounts of virus particles (4.09-4.92 $\log_{10}$ $TCID_{50}$/ml). The 3 rhabdovirus isolates were entering exponential phase of growth.
- At 7 h, the cell monolayers still looked normal. The free, swollen cells noted earlier seemed to have undergone cytolysis because of the presence of cell fragments in the medium and some new free, swollen cells had appeared. Amounts of cell-associated virus of isolates T9415 and T9429 were found to be greater than free virus.
- At 10 h, the infected cell monolayers started to show changes compared with the control cells. Cell nuclei began to condense. Both free virus and cell-associated virus values rapidly increased. The amounts of cell-associated virus for all isolates were found to be greater than free virus values.

- At 13 h, some cells with a condensed nucleus appeared in cell monolayers. More free, swollen cells and cell fragments were found in the medium. Amounts of virus rapidly increased to 6.50-7.92 $\log_{10}$ $TCID_{50}$/ml, which was near the end of the exponential growth phase (Figure 6.6). Values for cell-associated virus were still greater than for free virus.
- At 17 h, many small focal areas of change began to appear in comparison to FHM control cells (Figure 6.7a-b). Nuclei of cells in these areas were condensed and cell outlines were not clearly seen. The amounts of free virus of isolates T9415 and T9416 reached a maximum titre of 8.50 $\log_{10}$ $TCID_{50}$/ml as well as cell-associated virus at 8.09 and 8.25 $\log_{10}$ TCID50/ml. The cell-associated virus of isolate T9429 nearly reached a maximum level.
- At 22 h, the infected cell cultures were extensively damaged with 25% of the cell sheet affected. Many holes were present in the cell sheets and many rounded cells were floating free in the medium. Some rounded cells appeared 4-8 times larger than the normal cells. The amounts of free virus of isolates T9415 and T9416 began to stabilize at the maximum level, while isolate T9429 just reached maximum titre at 8.5 $\log_{10}$ $TCID_{50}$/ml. Values for free virus were greater than cell-associated virus. Growth of the 3 rhabdovirus isolates were in the stationary phase.
- At 28 h, 25-50% of cell sheets were affected. Dead cells had sloughed off the surfaces leaving clear areas on the plate (Figure 6.7c). Cells were shrunken and aggregated and showed loss of cell configuration. Vacuolation of infected cells spread through out the remaining cell sheet. Rounded cells and cell fragments were observed in the medium. Amounts of free virus of T9415, T9416 and T9429 remained greater than the cell-associated virus.

- At 35 h, 50%-75% of the cell sheets were affected. Changes were similar to 28 h but more extensive damage was observed.
- At 39.5 h, 75%-100% of the cell sheets were affected. Many cells had died and sloughed off the plate surfaces (Figure 6.7d). The remaining cells on the plate were shrunken and aggregated and showed loss of cell configuration. The amount of free virus of all isolates was generally higher than cell-associated virus.
- At 44-52 h, 100% of the cell sheets were affected. Most cells had died and sloughed off the surfaces. More T9429-infected cells remained attached to the plate surfaces than for the other isolates. The amount of free virus of isolate T9429 decreased 1 $\log_{10}$ $TCID_{50}$/ml during hours 52-60, while a 0.5 log increase was found in the amount of cell-associated virus.
- At 60-73 h, 100% of cell sheets were affected. All the T9415- and T9416-infected cells had died, sloughed off and lysed. The T9429-infected cells had a small number of cells remaining on the plate surfaces.

6.4.1.4 Stability at Low pH

Rhabdovirus isolates T9415, T9416 and T9429 were found to be sensitive to pH 3.0. The amounts of control virus incubated for 1 h in HBSS pH 7.2 were 8.25, 7.75 and 7.50 $\log_{10}$ $TCID_{50}$/ml, respectively, while the amounts of virus incubated in glycine-HCl pH 3.0 were 7.0, 6.50 an 5.75 $\log_{10}$ $TCID_{50}$/ml, respectively. Reductions of 1.25 $\log_{10}$, 1.25 $\log_{10}$ and 1.75 $\log_{10}$ $TCID_{50}$/ml indicated that the 3 new rhabdovirus isolates were sensitive to low pH.

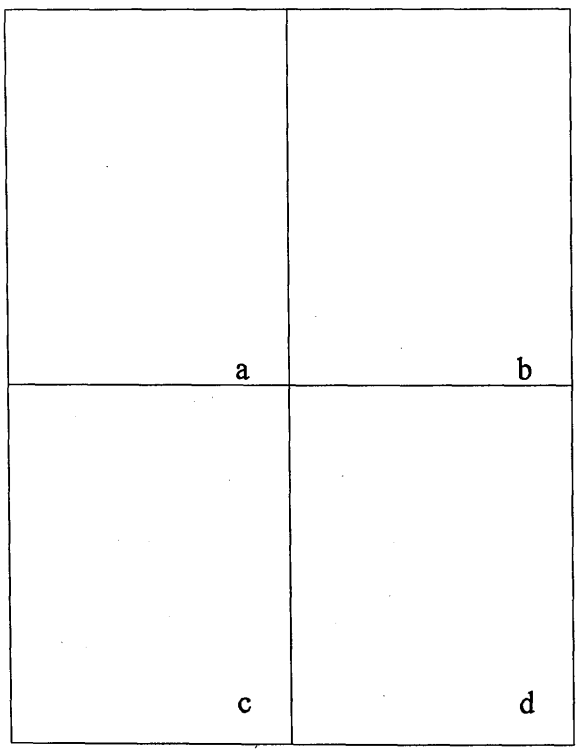
Figure 6.7 Progressive CPE in FHM cells after infection with rhabdovirus isolate T9415. Bar = 110 nm

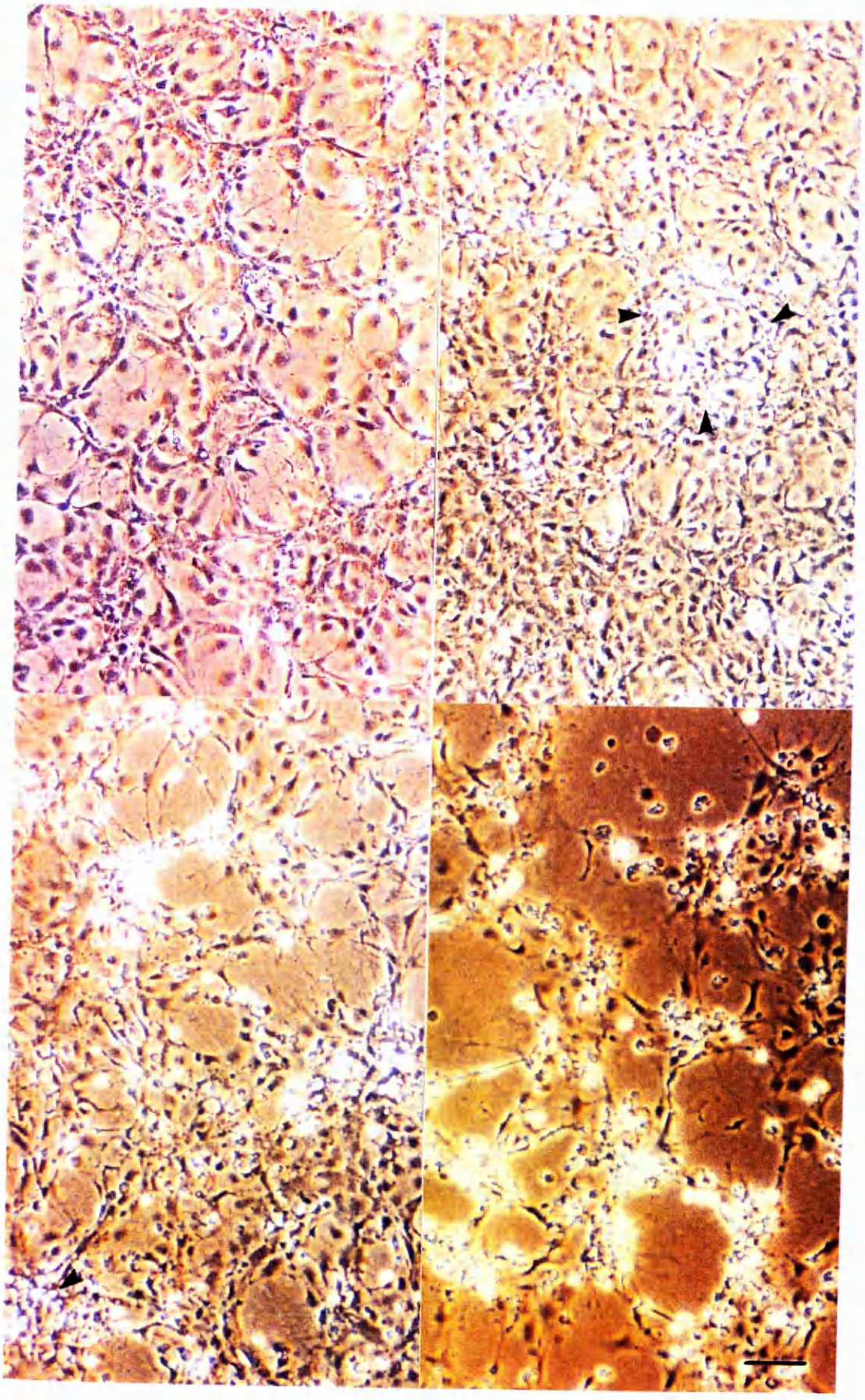
a. FHM control cells at 48 h showing normal monolayer appearance.

b. Infected FHM cells at 17 h showing many small focal changes (arrows) with condensed nuclei but the monolayer still intact. Maximum growth of virus was found at this stage.

c. Infected FHM cells at 28 h with 30% of monolayer area affected. The infected cells were vacuolated (arrows), shrunken, aggregated and showed loss of cell configuration.

d. Infected FHM cells at 39.5 h showing extensive damage with 90% of monolayer area affected. Most cells had died and sloughed off the surface.





6.4.1.5 Sensitivity to Heat

Rhabdovirus isolates T9415, T9416 and T9429 were extremely sensitive to heating at 56°C. Infectivities were entirely lost when incubated at 56°C for 30 min or 60 min, while control samples held at 4°C had 7.25, 7.25 and 7.0 $\log_{10}$ $TCID_{50}$/ml, respectively.

6.4.1.6 Sensitivity to Lipid Solvent

Rhabdovirus isolates T9415, T9416 and T9429 were extremely sensitive to the lipid solvent, chloroform. Infectivities were entirely lost when incubated with chloroform for 10 min at room temperature, while virus controls in HBSS held at 4°C had 8.50, 7.50 and 7.83 $\log_{10}$ $TCID_{50}$/ml, respectively. The chloroform solution was found to cause no harm to control FHM cells during 5 days observation.

6.4.1.7 Determination of Nucleic Acid Type

Rhabdovirus isolates T9415, T9416 and T9429 were resistant to IUDR compound. Virus titre for IUDR-treated samples were 6.50, 5.50 and 6.75 $\log_{10}$ $TCID_{50}$/ml, while the un-treated controls were 6.75, 6.25 and 7.0 $\log_{10}$ $TCID_{50}$/ml, respectively. Differences were less than 2 $\log_{10}$ $TCID_{50}$/ml for each virus indicating RNA type of nucleic acid. In comparison, IUDR-treated CCV virus had a titre of 5.5 $\log_{10}$ $TCID_{50}$/ml, while the un-treated control was 7.5 $\log_{10}$ $TCID_{50}$/ml. CCV showed a 2 $\log_{10}$ $TCID_{50}$/ml reduction indicating DNA type of nucleic acid.

6.4.1.8 Cell Line Susceptibility

Stocks of rhabdovirus isolates T9415, T9416 and T9429 propagated in the SSN-1 cell line in maintenance medium were tested for infectivity by titration in 10 cell lines in the same manner. Results are shown as a histogram in Figure 6.8. The SSN-1, HCK, HCT, BF-2 and FHM cell lines were susceptible to all 3 isolates. The SSN-1 line showed the highest sensitivity to infection with virus titres of 8.0, 8.25 and 7.5 $\log_{10}$ $TCID_{50}$/ml, respectively, while the FHM line showed the next best sensitivity with titres at 6.0-7.0 $\log_{10}$ $TCID_{50}$/ml. HCT supported growth to a lower degree at 2.5-3.5 $\log_{10}$ $TCID_{50}$/ml. BB and AS lines were sensitive to isolate T9416 only with virus titres at 6.26 and 4.75 $\log_{10}$ $TCID_{50}$/ml, respectively. Isolate T9416 gave the highest infectivity titre in every susceptible line. Three lines, EPC, CHSE-214 and RTG-2, were resistant to infection with all virus isolates.

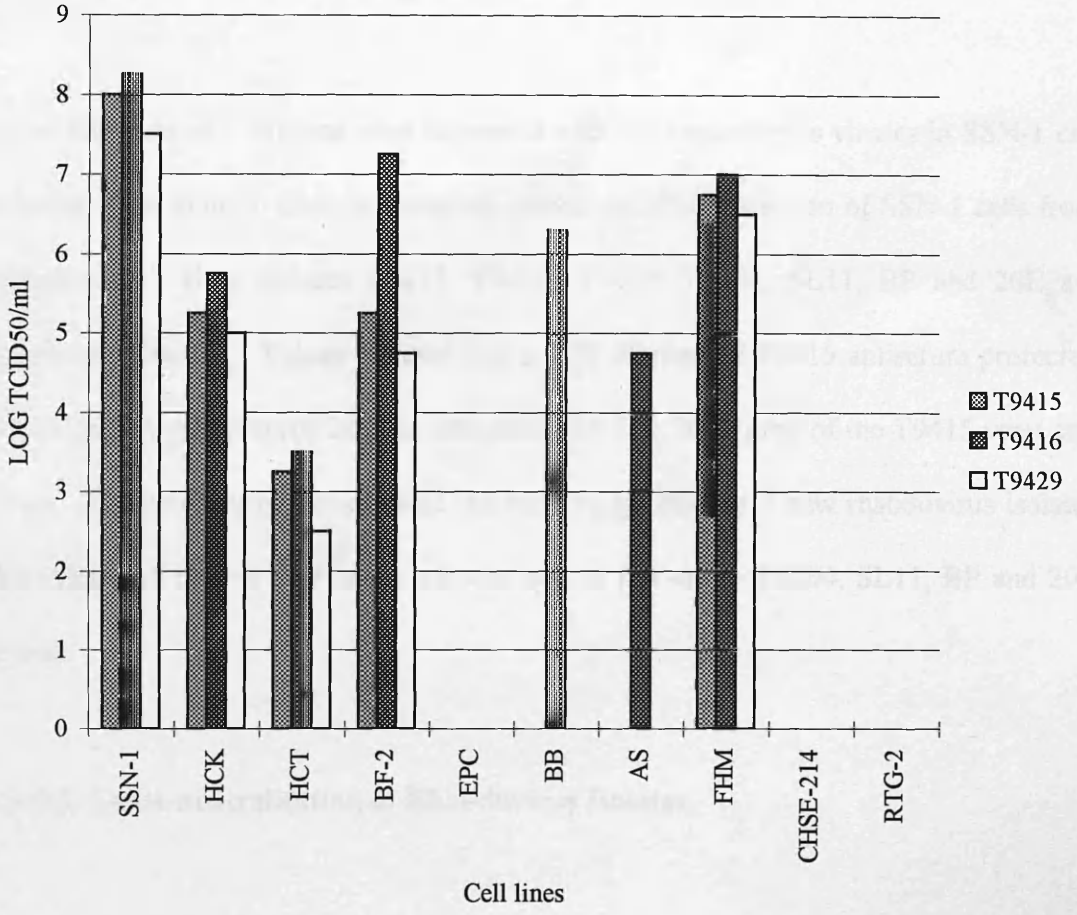


Figure 6.8 Cell line susceptibilities to rhabdovirus isolates T9415, T9416 and T9429 were determined by 50% end point infectivity assay. Stocks of all isolates were prepared in SSN-1 cells and titrated in 10 cell lines using 10-fold series dilutions and 4 replicate wells in 96-well plates. Incubation at 25°C except for CHSE-214 and RTG-2 which were incubated at 20°C.

6.4.2 Structural Proteins and Serological Comparions Between New Isolates and Previous Isolates of EUS-associated Rhabdoviruses

6.4.2.1 Titration of Neutralising Antisera

Serial dilutions of 7 antisera were incubated with the homologous viruses in SSN-1 cell cultures. The dilution of each antiserum providing 50% protection of SSN-1 cells from infection with virus isolates T9415, T9416, T9429, T9204, SL11, BP and 20E are shown in Table 6.6. Values indicate that a 1:20 dilution of T9415 antiserum protected half of SSN-1 cell cultures from an infection with 562 $TCID_{50}$/ml of the T9415 virus and so on. The neutralising activities of the antisera against the 3 new rhabdovirus isolates were found to be less than in the antisera against the earlier T9204, SL11, BP and 20E strains.

6.4.2.2 Cross-neutralisation of Rhabdovirus Isolates

Six antisera showed good neutralisation against the corresponding viruses with neutralisation indicies (N.I.) >2. An exceptionally low level of neutralisation was found for antiserum T9429 against the homologous virus with N.I. = 1. Each antiserum showed variable degrees of cross-neutralisation against 8 rhabdovirus isolates as shown in Table 6.7. Antisera T9415, T9416 and BP had the highest N.I. against the homologous viruses compared to the other antisera. Antisera T9429, T9204, SL11 and 20E neutralised other virus isolates better than the homologous viruses. Antiserum T9416 neutralised all 8 rhabdovirus isolates with a generally high N.I.

Table 6.6 Virus neutralisation of 7 rabbit antisera against 7 homologous rhabdovirus isolates in FHM cells. Dilutions of rabbit antisera giving 50% protection of SSN-1 cells from infection with the corresponding rhabdovirus isolate were recorded. Back titrations of the virus isolates are also included.

Rhabdovirus isolate	Antisera dilution at 50% protection of SSN-1 cells	Back titration of tested viruses ($TCID_{50}$/ml)
T9415	1:20	562
T9416	1:28	562
T9429	1:28	562
T9204	1:112	251
SL11	1:3548	1259
BP	1:40	1259
20E	1:447	2818

Table 6.7 Serum neutralisation between 7 rabbit antisera and 8 rhabdovirus isolates in FHM cells. The selected dilution of each antiserum is given in parentheses. Values indicate the neutralisation index (N.I.). N.I. equal to or greater than 1.7 indicates homologous or similar antigenicity.

Rhabdovirus isolate	Rabbit antiserum (dilution)						
	~T9415 (1:10)	~T9416 (1:20)	~T9429 (1:10)	~T9204 (1:40)	~SL11 (1:750)	~BP (1:15)	~20E (1:100)
T9415	**2.7**	2.3	2.3	4.0	0	0.7	0
T9416	1.3	**4.3**	1.7	2.0	0.3	0	1.0
T9429	1.0	2.0	**1.0**	1.0	0	0	0
SHRV	1.3	3.0	1.8	0.3	0	0	0
T9204	1.3	2.0	2.3	**2.3**	0	0	0
SL11	0	3.0	0	0	**4.2**	2.0	5.0
BP	0	3.7	0.8	0	5.3	**3.3**	6.0
20E	0	4.3	0	0	2.8	1.0	**4.7**

According to the N.I., the 8 virus isolates could be separated into 2 groups. The first group comprised isolates T9415, T9416, T9429, SHRV and T9204 and the second group contained 3 isolates, SL11, BP and 20E, as they shared some degree of neutralisation within their groups.

6.4.2.3 Analysis of Rhabdovirus Structural Proteins

Structural proteins of each virus isolate were separated using SDS-PAGE and the resulting gels were silver stained. The mobility patterns are shown in Figure 6.9. Molecular weights of protein bands were estimated (Table 6.8) by comparing their mobility to the standard curve of the markers (Appendix 3). According to the mobility patterns and molecular weights of the major protein bands, the isolates could be separated into 2 groups. The first group comprised isolates T9415, T9416, T9429, SHRV and T9204 for which 5 similar major protein bands were found. The molecular weights of the 5 major proteins were ~190, 63-66, 43-45, 29.5-30.0, and 22.0-23.5 kDa. The migration patterns and molecular weights were comparable to the L, G, N, M1 and M2 proteins of the *Lyssavirus* genus. Minor bands of molecular weight at 22.0-22.5 and 110-115 kDa were also observed for virus isolates T9415, T9416 and T9429. UDRV isolates SL11, BP and 20E were considered as the second group for which 4 major protein bands were found. Molecular weights of the 4 major proteins were ~190, 62-66, 45.5-48, and 26-27 kDa. The migration patterns and molecular weights were similar and closely related to the L, G, N and M proteins of the *Vesiculovirus* genus. Possible NS major protein bands with molecular weights ranging from

Table 6.8 Molecular weights of structural proteins of 8 isolates of rhabdovirus estimated from mobility curves of the standard markers. Mobility curves were plotted on a semilogarithmic scale (Appendix 3). Distances of protein migration in the gel were measured between the middle of the band and the top edge of resolving gel. Structural protein designations were determined according to Wagner *et al.* (1972) and McAllister and Wagner (1975).

Virus isolate	Molecular weights of structural protein (kDa)						
	L	G	N	(NS)*	M1	M	M2
T9415	190	65	44	–	30	–	23
T9416	190	66	45	–	30	–	23
T9429	190	65	44	–	30	–	23.5
SHRV	190	65	43	–	29.5	–	22.5
T9204	190	63	43	–	29.5	–	22
SL11	190	63	47	(44.5)	–	27	–
BP	190	66	48	(44.6)	–	26.5	–
20E	190	62	45.5	(44.5)	–	26	–

* NS structural protein possibly located below N protein band.

44.5-44.6 kDa were observed below the N protein band of UDRV isolates SL11, BP and 20E. Strains BP and 20E showed a few minor bands near the L protein that were not observed in the other 6 viruses. Isolate 20E contained additional minor bands at 18 kDa and 32 kDa.

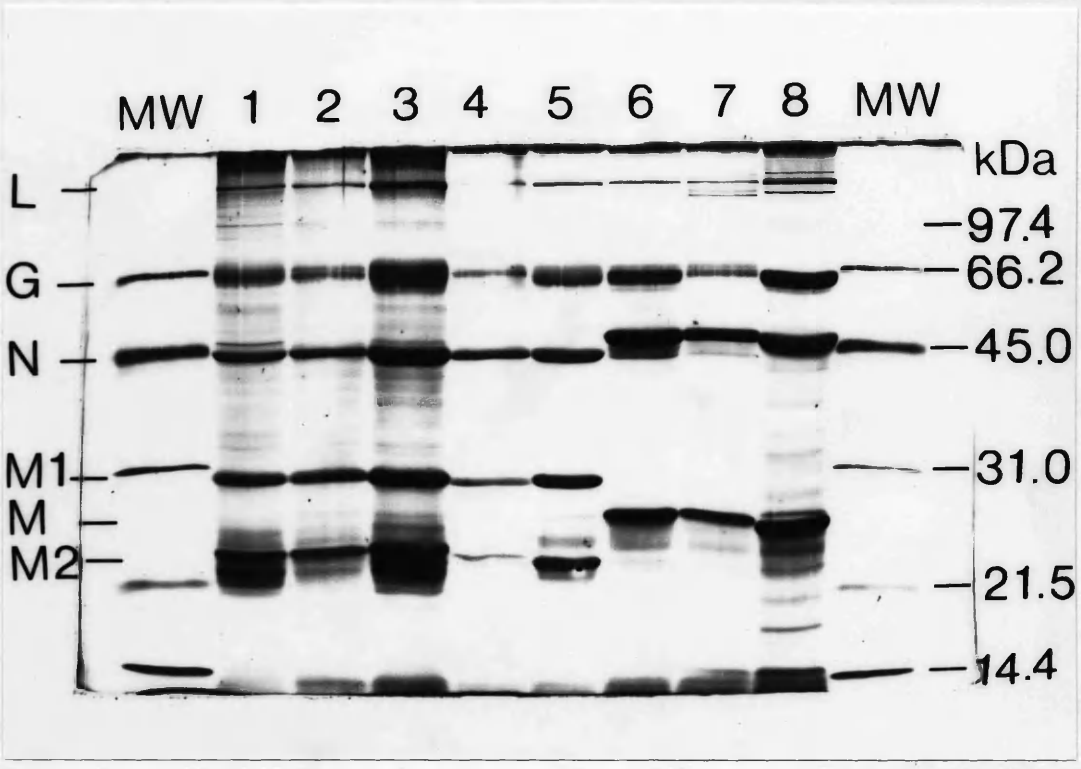
6.4.2.4 Western Blot Analysis of Rhabdovirus Isolates Using Rabbit Antisera

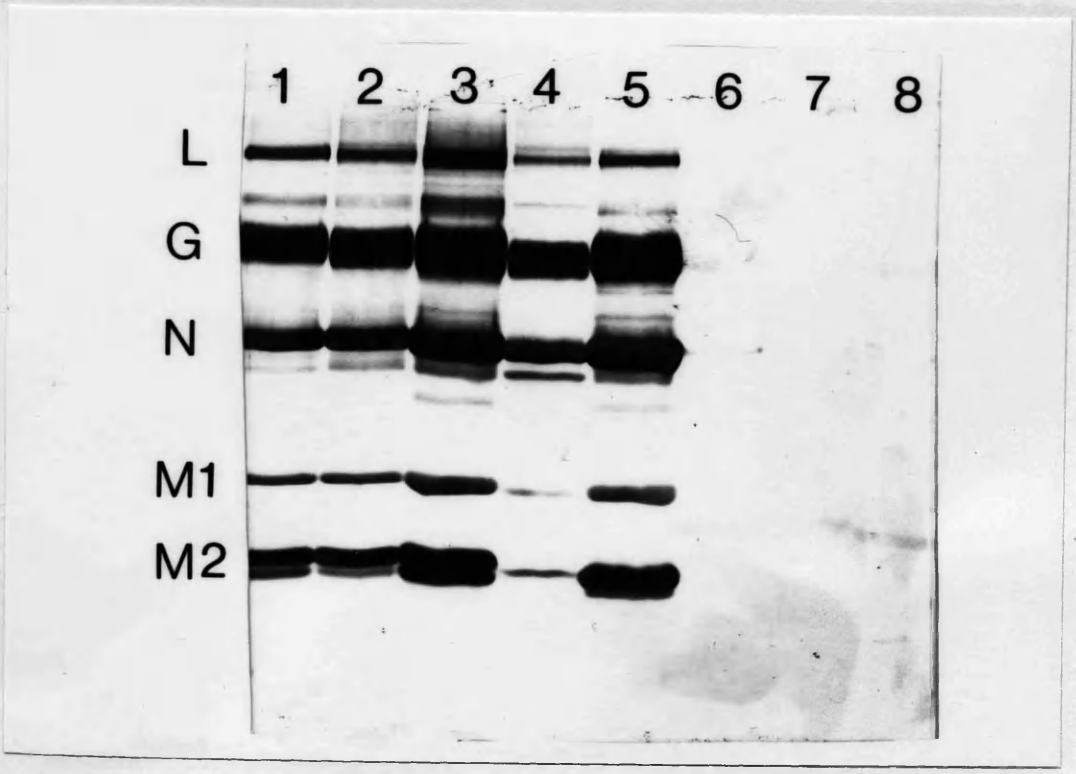
Antigenic identification profiles of 8 virus isolates were demonstrated by enzyme-linked immuno-staining on 5 blotting membranes with polyclonal antisera against rhabdovirus isolates T9415, T9416, T9429, T9204 and 20E. Molecular weight markers used in this study were at too low concentration to be stained with 0.1% amido black. Therefore, the immuno-stained bands were compared to the mobility patterns of the major protein bands (Figure 6.9). The first 4 polyclonal antisera immuno-stained all 5 major viral proteins of rhabdoviruses in lanes 1-5 while the fifth antiserum, 20E, only stained N, NS and M proteins of the UDRV group in lanes 6-8 as follows:

- Antiserum T9415 showed strong precipitation with structural protein bands in lanes 1-5 (Figure 6.10). The antiserum recognised all 5 major proteins of the homologous antigen (lane 1) and 4 heterologous antigens (lanes 2-5). Apart from the major bands, a minor band below M2 in lanes 1-3 and below N in lane 4 were also immuno-stained with antiserum T9415. There were very light precipitation bands G and M in lanes 6-8 of UDRV isolates SL11, BP and 20E.
- Antiserum T9416 showed the same strong precipitation with major protein bands in lanes 1-5 as antiserum T9415 (Figure 6.11). There was some degree of immuno-recognition by antiserum T9416 to viral proteins of isolates SL11, BP and 20E. The antiserum reacted with G, N and M proteins and a minor protein band at 33 kDa of these 3 viruses but to the lesser degree in comparison to the darker colour of major proteins in lanes 1-5. The N protein band showed a stronger antigenic similarity than

Figure 6.9 Mobility of structural proteins of 8 EUS-associated rhabdovirus isolates in 12% gel SDS-PAGE. Rhabdovirus allocations are follows: T9415 (lane 1); T9416 (lane 2); T9429 (lane 3); SHRV (lane 4); T9204 (lane 5); SL11 (lane 6); BP (lane 7); and 20E (lane 8). Molecular weight markers (lane MW) (BioRad, USA) contain 97.4 kDa phosphorylase B, 66.2 kDa serum albumin, 45.0 ovalbumin, 31.0 kDa carbonic anhydrase, 21.5 kDa trypsin inhibitor and 14.4 kDa lysozyme. The gel was stained with sliver staining kit (Sigma, UK). Rhabdovirus isolates T9415, T9416, T9429, SHRV and T9204 have 5 similar major protein bands which correspond to L, G, N, M1 and M2 major structural proteins of *Lyssavirus* while rhabdovirus isolates SL11, BP and 20E have 4 major protein bands which are similar to L, G, N, and M major structural proteins of *Vesiculovirus*. A possible NS major protein band is located below N protein in lanes 6-8.

Figure 6.10 Immuno-staining of antiserum T9415 against 8 rhabdovirus isolates on Western blot membrane. Rhabdovirus and proteins L, G, N, M1 and M2 allocations are comparable to those in Figure 6.9. Antiserum T9415 recognises all 5 structural proteins of rhabdovirus isolates T9415, T9416, T9429, SHRV and T9204. Low degrees of immuno-staining are found on G and M protein of rhabdovirus isolates SL11, BP and 20E.


MW 1 2 3 4 5 6 7 8 MW
kDa
L
G
N
M1
M
M2
97.4
66.2
45.0
31.0
21.5
14.4

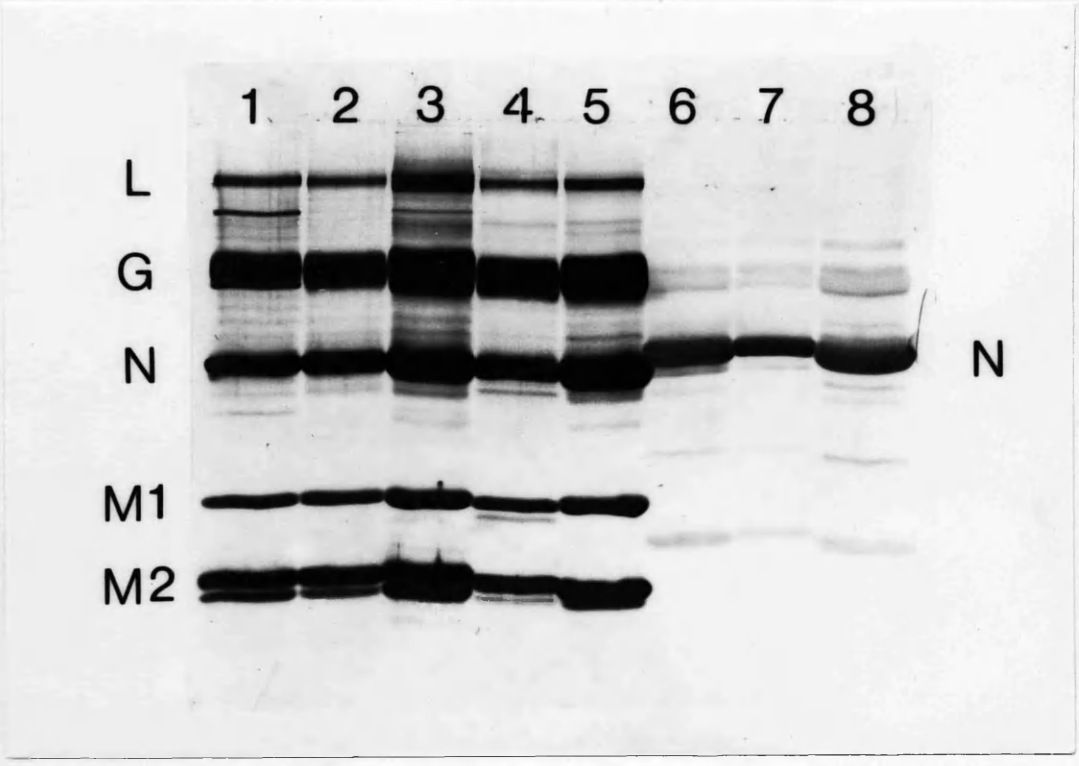

1 2 3 4 5 6 7 8
L
G
N
M1
M2

G, M, and 33 kDa protein bands. Precipitate band M2 in lanes 1-5 appeared as 2 bands close together when stained with antiserum T9416.

- With antiserum T9429, all 5 major protein bands in lanes 1-5 were stained similar to antisera T9415 and T9416 (Figure 6.12). There were low degrees of cross immuno-recognition to heterologous major proteins G and M in lanes 6-8. With antiserum T9204, the membrane showed a similar pattern of immuno-recognition to all 5 major proteins in lanes 1-5 as with antisera T9415, T9416 and T9429. A high degree of immuno-recognition to the G protein in lanes 1-8 was observed (Figure 6.13). A lesser degree of cross recognition to N and M protein bands were found in lanes 6 and 8.
- Antiserum 20E showed a contrasting result to the above 4 antisera. No protein bands in lanes 1-5 of isolates T9415, T9416, T9429, SHRV and T9204 were regcognised while strong immuno-recognition occurred with N, NS and M proteins in lanes 6-8 of UDRV isolates SL11, BP and 20E (Figure 6.14). The L and G proteins in lanes 6-8 were not clearly stained by antiserum 20E. The antiserum also stained a small protein band at 32-33 kDa in lanes 6-8.

Figure 6.11 Immuno-staining of antiserum T9416 against 8 rhabdovirus isolates on Western blot membrane. Rhabdovirus and proteins L, G, N, M1 and M2 allocations on the membrane are the same as Figure 6.9. Antiserum T9416 recognises all 5 structural proteins of rhabdovirus isolates T9415, T9416, T9429, SHRV and T9204. Variable degrees of immuno-staining are found on G, N, M and 32-33 kDa protein of rhabdovirus isolates SL11, BP and 20E.

Figure 6.12 Immuno-staining of antiserum T9429 against 8 rhabdovirus isolates on Western blot membrane. Rhabdovirus and proteins L, G, N, M1 and M2 allocations on the membrane are the same as Figure 6.9. Antiserum T9429 stains all 5 structural proteins of rhabdovirus isolates T9415, T9416, T9429, SHRV and T9204. Low degrees of immuno-staining are observed on G and M protein of rhabdovirus isolates SL11, BP and 20E.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G
N
M1
M2
N

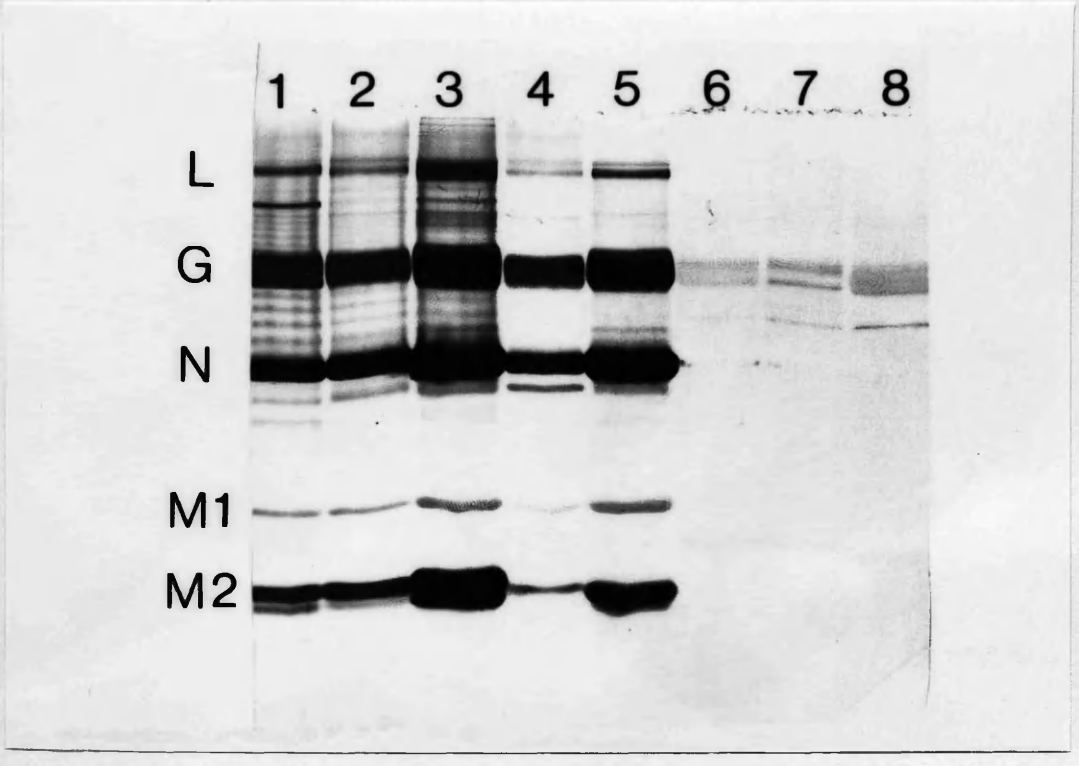
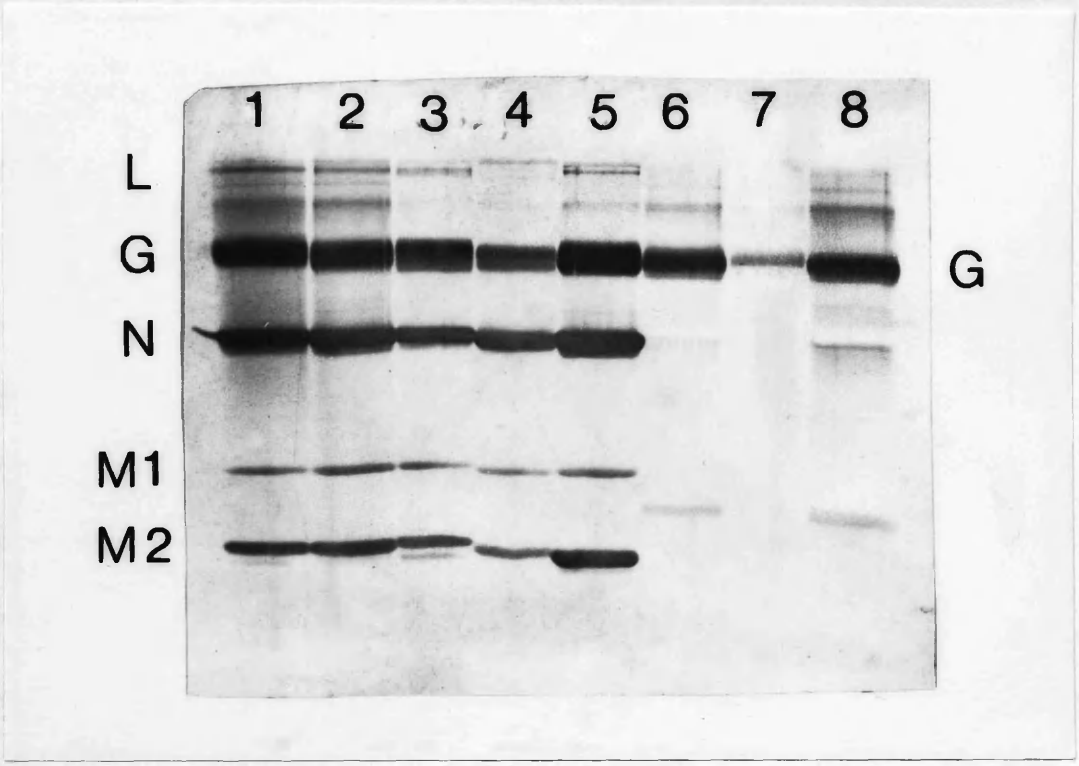

1 2 3 4 5 6 7 8
L
G
N
M1
M2

Figure 6.13 Immuno-staining of antiserum T9204 against 8 rhabdovirus isolates on Western blot membrane. Rhabdovirus and proteins L, G, N, M1 and M2 allocations on the membrane are similar to those of Figure 6.9. Antiserum T9204 recognises all 5 structural proteins of rhabdovirus isolates T9415, T9416, T9429, SHRV and T9204 and G protein of rhabdovirus isolates SL11, BP and 20E. Low degrees of immuno-staining are present on N and M proteins of rhabdovirus isolates SL11 and 20E.

Figure 6.14 Immuno-staining of antiserum 20E against 8 rhabdovirus isolates on Western blot membrane. Rhabdovirus and proteins N, and M allocations on the membrane are the same as Figure 6.9. Antiserum 20E recognises only N, NS and M structural proteins and 32-33 kDa protein of rhabdovirus isolates SL11, BP and 20E. NS protein is antigenic and located below N protein.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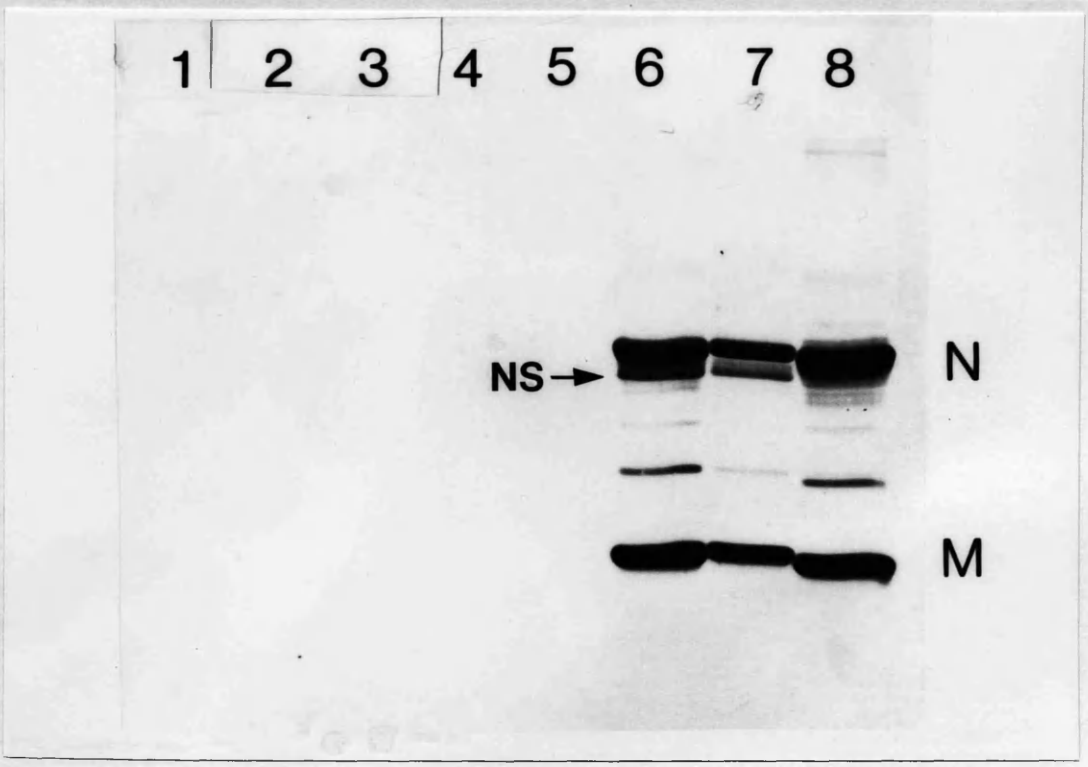
G
N
M1
M2
G


1 2 3 4 5 6 7 8
NS
N
M

6.4.2.5 Virus Neutralisation and Western Blot Analysis With Snakehead Fish Sera: Preliminary Observations.

Sixteen fish sera, collected from EUS-recovered snakehead fish, were tested for virus neutralisation against rhabdovirus isolate T9429. Eleven fish sera had no neutralisation activity while the other 5 sera from fish numbers 4, 5, 9, 11, and 15 seemed to have some degree of neutralisation. When cell cultures were observed on day 2 after inoculation, the dilutions of the 5 fish sera that protected SSN-1 cells from virus infection were 1/80, 1/160, 1/80, 1/40 and 1/40, respectively. On day 3 post-inoculation, the virus had caused further damage to the SSN-1 cells and there now appeared to be no protection from any of the 16 fish sera, even at the lowest serum dilution (1/10). Back titration of rhabdovirus isolate T9429 used in the virus neutralisation test was 3548 $TCID_{50}$/ml. Because only a low degree of neutralisation by the 5 fish sera was observed, the virus neutralising dilutions were presumed <1/10 (Table 6.9).

The results of immuno-staining with the 16 sera from EUS-recovered fish and 1 serum from a normal snakehead fish are shown in Figure 6.15 and details presented in Table 6.9. Lane 18 of the first blotting membrane was assigned for the 5 structural protein markers of T9429. Lanes 1-5, 8, 12 and 16 were stained too darkly to identify any precipitation bands. Only 8 lanes were used to record immuno-recognition bands. Seven out of eight lanes showed some degree of immuno-recognition by fish sera to rhabdovirus isolate T9429. Fish sera in lanes 6 and 7 recognised M2 protein. There was an un-designated precipitation band between G and N protein markers which

Table 6.9 Virus neutralisation and immuno-blotting of EUS-recovered snakehead fish sera against rhabdovirus isolate T9429. Recovered fish were caught from the disease pond 2 months after the outbreak. T9429 was also isolated from the diseased fish in this pond. Neutralisation tests were performed in SSN-1 cells in 96-well plates. Amount of virus used in the test was 3548 $TCID_{50}$/ml. Immuno-recognition of surviving fish sera are recorded.

EUS-recovered snakehead fish			Serum dilution	Western blot 1		Western blot 2	
Fish serum	Weight (g)	Healing wound	caused neutralisation	Lane no.	Immuno recognition	Lane no.	Immuno recognition
1	760	yes †	–	1	*	2	–
2	175	yes	–	2	*	3	–
3	202	yes	–	3	*	4	–
4	600	yes	<1/10	4	*	5	M1
5	600	yes	<1/10	5	*	6	–
6	275	no	–	6	M2, ‡	7	–
7	540	no	–	7	M2	8	–
8	480	yes	–	8	*	9	–
9	500	yes	<1/10	9	M1	10	–
10	170	yes	–	10	–	11	–
11	280	yes	<1/10	11	M1	12	M1
12	510	no	–	12	*	13	M1
13	250	no	–	13	M1	14	–
14	160	yes	–	14	M1	15	–
15	400	no	<1/10	15	M1	16	–
16	210	yes	–	16	*	nd	nd
17 ¶	500-600	–	–	17	M1	17	–

† 'yes' indicates that the recovered fish showed healing wounds or disfigured body; 'no' indicates normal appearance.

* Lanes on the membrane (Figure 6.15a) were too dark to identify immuno-recognition.

‡ An un-designated precipitation band located between proteins G and N (Figure 6.15a).

¶ Fish serum 17 was pooled sera of normal snakehead fish stocking at AAHRI. The fish were originally brought from Amphur Song Pee-Nong, Suphanburi province in August, 1993.

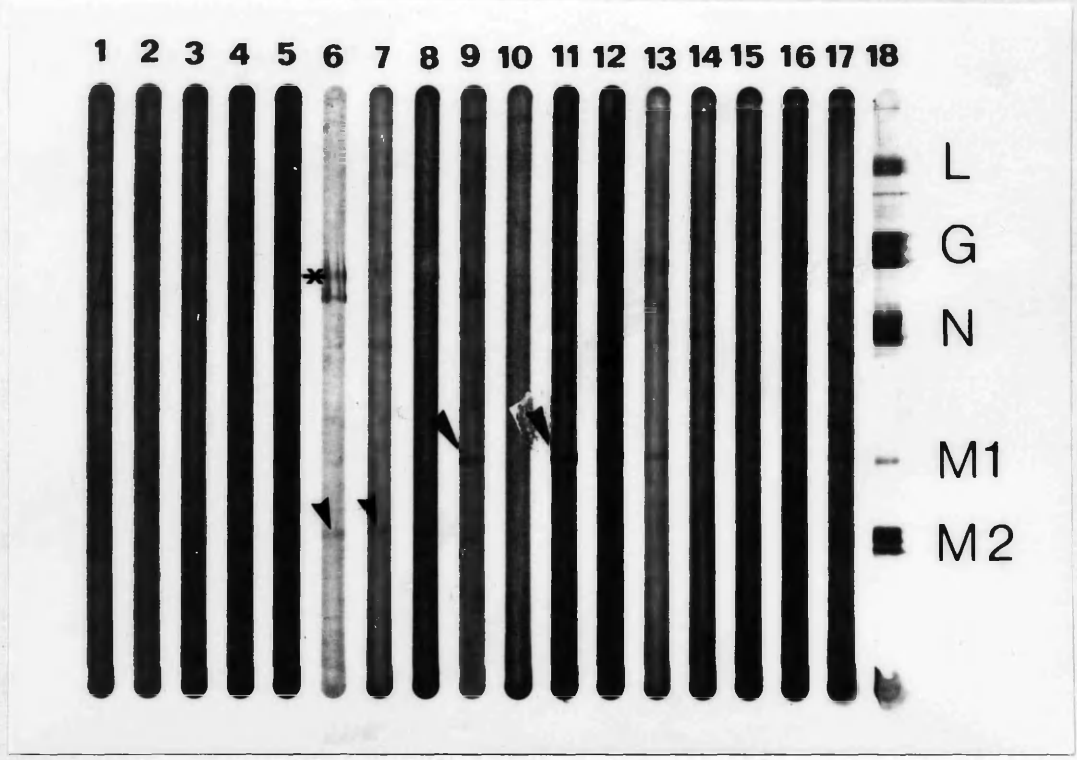
nd = not done.

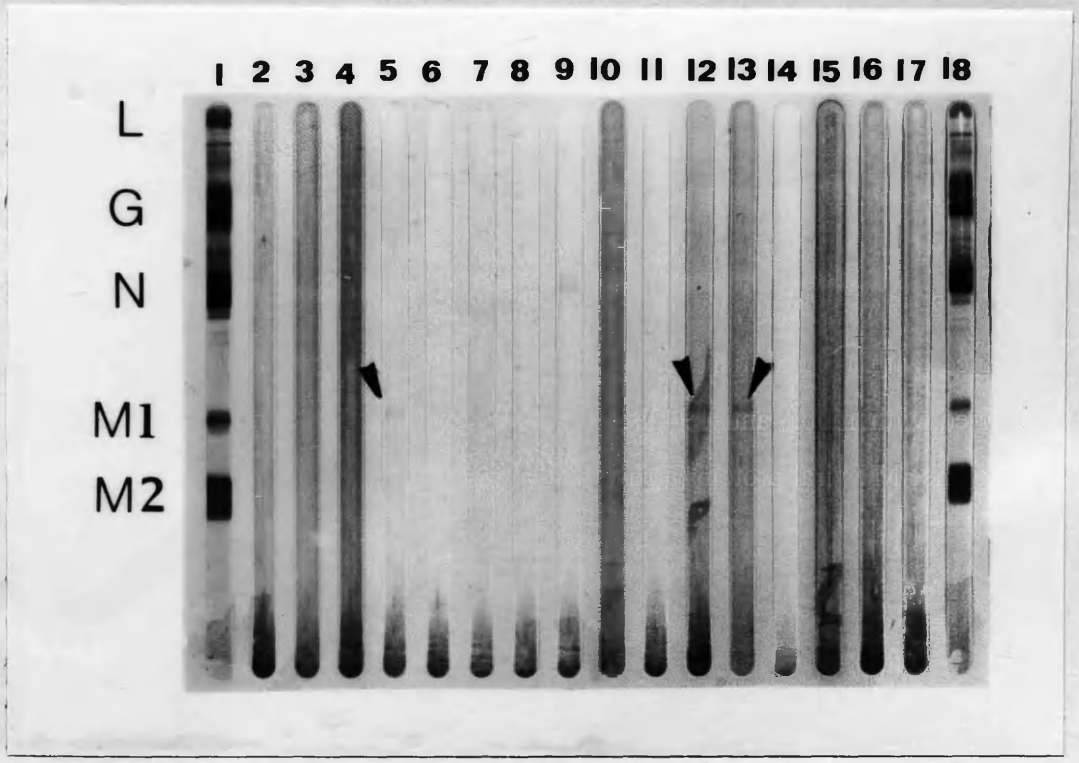
immuno-stained with fish serum 6. Sera in lanes 9, 11, 13, 14 and 15 recognised M1 protein antigen but no immuno-recognition was observed in lane 10. The normal fish serum in lane 17 also gave positive immuno-staining to M1 protein on the first blotting membrane (Figure 6.15a). For the second immuno-blotting membrane, the same fish sera were applied to the membrane except for serum 16. Lanes 1 and 18 were assigned as reference locations for viral protein bands. The new layouts on the membrane for 15 sera from EUS-recovered fish and 1 serum from normal fish are indicated in Table 6.9. Applying different immuno-staining protocols, all 16 lanes showed a clearer background than the first membrane. However, only 3 out of 15 (20%) sera were found to recognise viral protein band M1 (Figure 6.15b). There was no immuno-stained band from the normal fish serum on this blotting membrane.

Figure 6.15 Immuno-staining of fish sera against rhabdovirus isolate T9429 on Western blot membranes. Markers L, G, N, M1 and M2 are comparable to those of Figure 6.9.

a. Non-specific sites on the membrane were blocked with 1% milk powder in TBS. Fish sera were diluted 1:10 and incubated with the membrane for 1 h at room temperature. Lanes 1-16 were incubated with different EUS-recovered fish sera and lane 17 was incubated with normal fish serum. Lane 18 was control viral proteins incubated with rabbit anti-T9429. Most fish sera recognise M1 viral protein (long arrows) while 2 fish sera recognise M2 viral protein in lanes 6 and 7 (short arrows). An un-designated band (*) is stained with fish serum 6.

b. Non-specific sites on the membrane were blocked with 3% gelatine in TBS. Fish sera were diluted 1:40 and incubated with the membrane for 24 h at 20°C. Lanes 2-16 were stained with different EUS-recovered fish sera and lane 17 was stained with normal fish serum. Lanes 1 and 18 were control viral proteins stained with rabbit anti-T9429. Fish sera in lanes 5, 12 and 13 were able to recognise M1 viral protein (arrows).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
L
G
N
M1
M2


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
L
G
N
M1
M2

6.5 Discussion

Rhabdovirus isolates from snakehead fish (T9415) and three-spot gourami (T9416), collected from Bangkok, and from snakehead fish (T9429), collected from Suphanburi province, had similar bullet-shaped morphology. The sizes of the 3 isolates in ultra-thin sections were similar with mean dimensions ranging from 174-177 × 48-53 nm (length × diameter). Dimensions of negative-stained pelleted virus particles of all isolates had wider mean ranges of 180-215 × 58-71 nm. Projecting spikes were ~10 nm and were seen in some pelleted virus preparations and a few virus particles in ultra-thin section preparations. Castric and Chastel (1980) also noted that the projecting spikes in European eel rhabdovirus were not easily seen in either virus pellet or section preparations. The diameter of the viruses in both types of preparation seemed similar if the length of the missing spikes was included. Particle lengths were greater in pelleted virus preparations as the full length of the virions were measured. The fish rhabdoviruses of this present study contain about the same number of neucleocapsid coils (~35 coils) as vesicular stomatitis virus (VSV) that infects higher vertebrates and insects (Dubois-Dalcq *et al.*, 1984). A rhabdovirus-like particle found in the muscle tissue next to the clinical lesion of a three-spot gourami was ~209 × 81 nm in size, which is about the size of typical rhabdovirus, but only 1 particle was found in the whole section. Some difficulties were encountered in trying to compare the sizes of these 3 new isolates with other EUS rhabdoviruses, which may arise due to different EM preparation protocols or measurements. Ahne *et al.* (1988) reported the size of SHRV at 170 × 60 nm, whereas the same strain was measured at 180-200 × 60-70 nm by Kasornchandra *et al.* (1991). The first report of UDRV was 120 ± 10 × 80 ± 5 nm in

dimensions for a pelleted virus preparation (Frerichs *et al.*, 1986) but 110-130 × 50-70 nm in an ultra-thin section preparation (Lilley and Frerichs, 1994). Tissue section preparation for transmission electron microscopy caused a reducing effect on virus dimensions, which has also been recognised for other fish rhabdoviruses (Darlington *et al.*, 1972).

The 3 new rhabdovirus isolates lost infectivity entirely when incubated at 56°C for 30 min. As is typical for rhabdoviruses, all isolates were sensitive to lipid solvent indicating a virus with a lipid containing envelope. As shown by IUDR treatment, all isolates possessed a RNA genome as this deoxyuridine analogue had no effect on virus multiplication (Rovozzo and Burke, 1973). All isolates were sensitive to glycine-HCl pH 3 as reductions of 1.25-1.75 $\log_{10}$ $TCID_{50}$/ml were observed following exposure for 1 h. These biochemical properties are in general agreement with other fish rhabdoviruses but variations may occur with different viruses and/or different test methods. Kasornchandra *et al.* (1991) used a different protocol for pH 3 sensitivity testing and found that SHRV was completely inactivated by 1 h treatment. Complete loss of infectivity was also reported for pH 3-treated SVCV (spring viremia of carp virus) (Fijan *et al.*, 1971). However, 1.75-2.0 $\log_{10}$ $TCID_{50}$/ml reductions, which were similar to this study, were reported for pH 3-treated HRV (hirame rhabdovirus) for 3 h (Kimura *et al.*, 1986). The partial characterisation of the 3 new rhabdovirus isolates indicates that all isolates belong to the family Rhabdoviridae.

The FHM cell line has the ability to grow in medium L-15 over the temperature range 14-34°C (Gravell and Malsberger, 1965) and is susceptible to a wide range of viruses

including homeiothermic viruses (Solis and Mora, 1970). The virus growth experiments conducted at 15-30°C were therefore compatible with the maintenance of FHM cells. Rhabdovirus isolates T9415 and T9416 had very similar growth patterns in FHM cells which were significantly slower at 15°C when compared to growth at 20, 25 and 30°C (p = 0.01 – 0.02). Slower growth at 15 °C than at 20-30°C was also reported for UDRV strains BP and 19 in SSN-1 cells (Frerichs *et al.*, 1989b). In contrast, the growth of Suphanburi isolate T9429 at 15°C was similar to growth at 20-30°C (p = 0.39). Rhabdovirus isolate T9429 seemed to grow better at 15°C than isolates T9415 and T9416 but statistical analysis showed non-significant differences ($p > 0.05$). However, 25-30°C was the optimum growth temperature for the 3 isolates. Similar wide ranges of growth temperatures with high optimum temperatures were also reported for EUS-associated UDRV (Frerichs *et al.*, 1989b) and SHRV (Kasornchandra *et al.*, 1991). The growth ability of these rhabdoviruses at high temperature is a typical characteristic of warm-water fish viruses (Wolf and Mann, 1980).

The 3 new rhabdovirus isolates had almost identical ($p > 0.05$) one-step growth curves in FHM cells at 25°C. there was approximately a 2 h latent period in which no new free progeny virus was detected in the supernatant fluid. Free virus appeared to be released after 2 h as high amounts of virus particles associated with the cell membrane were detected at this time. Most cell-associated virus particles are believed to be complete virions which remaining attached to cell fragments following 3× freeze-thaw cycles. It was noted that isolates T9415, T9416 and T9429 decreased in titre by 0.67, 0.83 and 1.0 $\log_{10}$ $TCID_{50}$/ml, respectively, following 3× freeze-thaw cycles. A similar reduction, 0.9 $\log_{10}$, was also reported for SHRV by Kasornchandra *et al.* (1991).

The results of the present study showed that new rhabdovirus progeny started to be released from infected FHM cells between 2-4 h post-incubation. The viruses then continued to increase exponentially until reaching a stationary phase at 13-17 h for cell-associated virus and 17-22 h for free virus. During the exponential phase, the amount of free virus seemed to be less than that of cell-associated virus, but the amount of free virus seemed to be greater when the stationary phase was reached. Surprisingly, when the amount of rhabdovirus had reached a maximum, the infected FHM cells showed only small focal changes in the monolayers. Periods of latent, exponential and stationary growth of the new isolates were very similar to the growth of EUS-associated SHRV in EPC cells at 27°C (Kasornchandra *et al.*, 1991) which also originated from the same geographical area of central Thailand. For cold-water fish rhabdoviruses, the latent periods seemed a little longer. The latent period of IHNV strain OSV in CHSE-214 cells at 18°C was reported as 4 h (McAllister *et al.*, 1974). Mulcahy *et al.* (1984) compared the growth characteristics in EPC cells of 10 IHNV strains, originating from different geographical areas between California and Alaska, at 15°C and 18°C and found that the latent periods of different strains varied between 5-20 h post-infection with shorter times at higher incubation temperatures. During the development of rhabdovirus virions, viral neucleocapsid protein was first synthesised at 1-2 h post-infection for the homoiothermic vesicular stomatitis virus (Wagner *et al.*, 1970), at 2-3 h for IHNV (Hsu and Leong, 1985), at 4-6 h for VHSV and at 6-8 h for HRV (Nishizawa *et al.*, 1991a). Employing an immuno-fluorescent staining technique, viral protein antigens could be visualised and were seen to be synthesized in the cytoplasm of infected cells during the latent and exponential phases of growth of fish rhabdovirus (McAllister *et al.*, 1974).

Surprisingly, rhabdovirus isolate T9416 showed a different cell line susceptibility pattern to isolates T9415 and T9429. Isolate T9416 was the only tested virus which grew in BB and AS lines at 25°C. These 2 cell lines reportedly supported the growth of many strains of UDRV (Lilley and Frerichs, 1994) which may indicate some degree of similarity between isolate T9416 and UDRV. Lines HCK, HCT, BF-2, FHM and SSN-1 were susceptible to infection with all 3 isolates, while EPC, CHSE-214 and RTG-2 were refractory. The refractory nature of EPC cells against EUS-associated virus infection was observed earlier (Chapter 3). Apart from SSN-1, which was used to grow stocks of all viruses, FHM cells showed the best support for the growth of all the new isolates. Virus titrations in 5 susceptible cell lines gave similar patterns with highest titres for isolate T9416 and lowest titres for isolate T9429. Maximum yields were recorded in the SSN-1 cell line at 7.5-8.28 $\log_{10}$ $TCID_{50}$/ml. However, in one-step growth studies of the isolates, in which stock viruses had been prepared in FHM cells and then titrated in the same cell line, the maximum titers were 8.5 $\log_{10}$ $TCID_{50}$/ml. Cell line susceptibility tests clearly separated isolate T9416 from T9415 and T9429 as a different strain. UDRV strains 02, 04, BP, 19 and 20 also showed different infectivity patterns in 12 cell lines (Frerichs *et al.*, 1989a). SSN-1 and BF-2 lines were susceptible to all 5 strains, while CHSE-214, RTG-2 and EPC lines were refractory, which was similar to the present study. Interestingly, the EPC line was used to grow SHRV (Ahne *et al.*, 1988; Kasornchandra *et al.*, 1991) but was refractory to the rhabdovirus isolates of this study and to all strains of UDRV (Frerichs *et al.*, 1989a). This information may indicate a distinct characteristic of the strain SHRV or the EPC line maintained at different

laboratories might have different properties that cause a variable sensitivity to virus infection.

Rhabdovirus isolates T9415, T9416, T9429, SHRV and T9204 possess a lyssavirus-like electrophoreotype while UDRV-SL11, -BP and -20E possess a vesiculovirus-like electrophoreotype. Protein profiles of the 5 lyssavirus-like rhabdoviruses were very similar with molecular weights ~190, 63-66, 43-45, 29.5-30.0 and 22-23 kDa corresponding to proteins L, G, N, M1 and M2, respectively. The 3 vesiculovirus-like rhabdoviruses were also similar within the profile type with molecular weights ~190, 62-66, 45.5-48.0 and 26-27 kDa which related to proteins L, G, N and M, respectively (Wagner *et al.*, 1972; McAllister and Wagner, 1975). Protein NS was not clearly seen in silver stained gel but it was possibly located below the N protein with a size of 44.5-44.6 kDa on Western blot membranes. These findings are in general agreement with the protein localisation patterns of SHRV and UDRV-BP reported by Kasornchandra *et al.* (1992). These authors observed that protein NS of UDRV migrated faster than protein N and found glycosylation only in protein G of both SHRV and UDRV. A similar localisation of NS protein was reported for *Rhabdovirus anguilla* strains EVA and EVX (EVEX) (Hill *et al.*, 1980; Castric *et al.*, 1984). In contrast to other vesiculovirus-like fish rhabdoviruses, NS proteins located above the N protein indicating a larger molecular weight have been reported for SVCV and PFRV (Lenoir and de Kinkelin, 1975; Clerx and Horzinek, 1978), European lake trout rhabdovirus (Björklund *et al.*, 1994) and pike rhabdovirus DK 5533 (Jørgensen *et al.*, 1993). EUS-associated rhabdoviruses and eel rhabdoviruses (Castric *et al.*, 1984) have one similar characteristic in that the viral proteins of both groups show 2 electrophoreotype patterns in SDS-PAGE.

Although rabbit antisera to 7 rhabdoviruses were prepared in a similar manner,. the neutralising activity obtained was variable. The antisera to all 3 new isolates examined in this study had very low neutralising levels compared to the 4 previous antisera. These findings may indicate variation in antigenic expression of different rhabdovirus isolates in the rabbit or that the rabbit immunisation protocol used may be less suitable for lyssavirus-like EUS-associated rhabdovirus. However, low neutralising activity and inconsistent antiserum production have also been reported for SVCV, PFRV, IHNV and VHSV fish rhabdoviruses, but not *Rhabdovirus anguilla* strains EVA and EVX (Hill *et al.*, 1981).

Serum neutralisation tests with UDRV-SL11, -BP and -20E antisera against 8 rhabdoviruses showed cross-reactions only within the vesiculovirus-like rhabdovirus group indicating similar serotypes. The T9415, T9429 and T9204 antisera cross-neutralised the viruses within the lyssavirus-like rhabdovirus group. Variable degrees of cross neutralisation may have been due to low neutralising antibody titres of this second group of antisera and toxic effects which were found at dilutions less than 1:10 for antiserum T9429 and 1:40 for antiserum T9204. There is a need to produce new antisera to isolates T9415, T9416, T9429, T9204 and SHRV and repeat the cross-neutralisation study to provide better comparisons. Earlier reports had shown no cross-neutralisation between the 2 electrophoreotype groups of EUS-associated rhabdoviruses (Kasornchandra *et al.*, 1992; Lilley and Frerichs, 1994). Surprisingly, in the present study, antiserum T9416 showed cross neutralisation to both lyssavirus- and vesiculovirus-like EUS-associated rhabdoviruses with high N.I. values. However, no

cross neutralisation was reported for eel viruses which also grouped as lyssavirus- and vesiculovirus-like rhabdoviruses (Castric and Chastel, 1980; Castric *et al.*, 1984).

The 5 structural proteins of lyssavirus-like EUS-associated rhabdovirus isolates T9415, T9416, T9429 and T9204 were antigenic and induced antibody responses in the rabbit. Western blot analysis with these 4 antisera revealed precipitation bands which corresponded to the 5 structural proteins of homologous and heterologous viruses within the lyssavirus-like group. Antiserum 20E clearly showed antigenic recognition within the vesiculovirus-like EUS-associated rhabdoviruses for structural proteins N, NS and M but no reaction with viral proteins of the 5 lyssavirus-like rhabdoviruses. A faint minor protein band for vesiculovirus-like rhabdoviruses at 32-33 kDa in SDS-PAGE was antigenic to rabbits and showed up on the immuno-stained membrane. This minor protein had no clear designation but was possibly a degraded structural protein. Although there was an awareness that this minor protein band might have originated from retrovirus which was simultaneously produced from SSN-1 cells and had about the same buoyant density in sucrose gradients as rhabdovirus (Frerichs *et al.*, 1991), this was unlikely as the protein was also recognised by antiserum T9416. Antiserum T9416 had been prepared from rhabdovirus isolate T9416 which had never been cultured in SSN-1 cells or other retrovirus infected cell line. Western blot analysis with other fish rhabdoviruses using homologous polyclonal antiserum also identified 5 protein bands in IHNV (Engelking and Leong, 1989) but 4 protein bands G, N, M1 and M2 in VHSV (Lorenzen *et al.*, 1988) and HRV (Nishizawa *et al.*, 1991b).

A further finding with no clear explanation was the absence of precipitated glycoprotein for UDRV-SL11, -BP and -20E immuno-stained with antiserum 20E. Glycoprotein is a well known viral antigen of both *Vesiculovirus* and *Lyssavirus* which gives rise to the serotype-specific neutralising antibody response in warm-blooded animals (Cox *et al.*, 1977; Dietzschold *et al.*, 1974; Kang and Prevec, 1970; Kelly *et al.*, 1972; Wiktor *et al.*, 1973). The lyssavirus-like fish rhabdovirus IHNV contains glycoprotein which gave rise to rabbit antiserum with similar properties to those of higher vertebrate rhabdoviruses (Engelking and Leong, 1989; Engelking *et al.*, 1991; Huang *et al.*, 1994). Glycoprotein of lyssavirus-like rhabdovirus also elicited neutralising and protective antibody in fish (Engelking and Leong, 1989; Lorenzen *et al.*, 1993). The properties of mono-specific glycoprotein polyclonal antisera of vesiculovirus-like fish rhabdoviruses have not yet been reported, but it is likely to be similar to those of higher vertebrate rhabdoviruses and lyssavirus-like fish rhabdoviruses. Western blot analysis of structural proteins of vesiculovirus-like rhabdoviruses SVCV and PFRV showed precipitate protein bands G, NS, N and M when immuno-stained with homologous antiserum (Jørgensen *et al.*, 1989). Although the glycoprotein of the 3 UDRV isolates was not recognised by polyclonal antiserum 20E, they were nevertheless strongly recognised by antiserum T9204 and partially recognised by antisera T9416 and T9429 instead. As antiserum 20E had very strong neutralisation activity (N.I. = 4.7-6.0) against the 3 UDRV isolates, it was believed to contain specific antibody to the surface projecting spikes (G protein). All antigenic epitopes on the G protein of vesiculovirus-like UDRV might have an entirely changed structural conformation after SDS-PAGE and Western blotting resulting in no precipitation band, while some antigenic epitopes of the G protein of the 5 lyssavirus-like rhabdoviruses were still retained and appeared as immuno-stained band

G on the blotting membrane. The changed structural conformation might have exposed some common non-neutralising epitopes of EUS-associated rhabdovirus which were regconised by lyssavirus-like rhabdovirus T9204 antiserum. Interestingly, the polyclonal antiserum to lyssavirus-like SHRV was also able to recognise protein G of vesiculovirus-like UDRV, SVCV, PFRV and lyssavirus-like IHNV, VHSV, HRV in Western blot membranes while no cross-neutralisation was reported for these fish rhabdoviruses (Kasornchandra *et al.*, 1992). VHSV was also serologically distinct from carpione rhabdovirus but its polyclonal and N-specific monoclonal antisera were also able to immuno-stain G and N proteins, respectively, of carpione rhabdovirus (Bovo *et al.*, 1995). The antigenic properties of lyssavirus-like SHRV were studied extensively using monoclonal antibody (MAb) (Kasornchandra *et al.*, 1992). Eight MAbs were able to form an immuno-precipitate band at protein G but only 3 MAbs could neutralise SHRV. Therefore, antiserum T9204 might recognise only non-neutralising epitopes of protein G of vesiculovirus-like UDRV which showed no serum cross-neutralisation but was detected by Western blot analysis.

Cross-neutralisation between 2 electrophoreotypes of rhabdovirus has never been found with salmonid and non-salmonid fish rhabdoviruses or warm-blooded animal rhabdoviruses but it appeared in EUS-associated isolate T9416. Interestingly, the cross antigen-antibody reaction complex of isolate T9416 appeared stronger for protein N than proteins G and M of vesiculovirus-like UDRV in Western blot membrane. However, it has been clearly established that N protein of lyssavirus-like fish rhabdoviruses does not elicit neutralising antibody in fish (Engelking and Leong, 1989) and N protein of prototype *Lyssavirus* and *Vesiculovirus* do not give rise to neutralising

antibody in warm-blooded vertebrates (Wiktor *et al.*, 1973; Dietzschold *et al.*, 1974). Therefore, failure of infectivity of antiserum-treated UDRV should be caused by the blockage of projecting spikes according to the general properties of rhabdovirus. To clarify this unusual cross serum neutralisation, mono-specific antisera to structural proteins of isolate T9416 need to be produced and examined for neutralising ability against homologous virus and UDRV.

The preliminary studies on virus neutralisation and Western bolt analysis with EUS-recovered fish sera gave an indication that the recovered snakehead fish had developed a humoral immune response to rhabdovirus. This is the first immunological study with naturally recovered snakehead fish sera against rhabdovirus. A few drawbacks occurred with the materials used in the tests. Fish sera had been kept very long (>1 year at -70°C). They were sent abroad from AAHRI to the Institute of Aquaculture. Unfortunately, the amount of dry ice was not enough to keep the samples cool throughout transportation, some sera went turbid with a strong fishy smell and only one control normal fish serum sample was available for the test. Rabbit anti-snakehead fish Ig antiserum obtained from the Immunology unit, AAHRI, had not been tested for antibody titre. The improper handling of fish sera and a labile nature of fish antisera may have decreased neutralising antibody levels so that no fish sera were able to protect SSN-1 cells from infection with rhabdovirus. However, some fish sera were able to slow the rate of virus infection indicating a possible low level of virus neutralisation. Western bolt analysis involved fish antisera, rabbit anti-fish Ig and donkey anti-rabbit IgG. Due to the limited supply of fish sera and sucrose gradient-purified rhabdovirus T9429, the test system was unable to be optimised for antisera dilutions. Only 2 runs of the Western

blot were obtained with poor immuno-staining. Many recovered fish had antibody to viral proteins M1 and a few fish showed an antibody response to part of viral protein M2. A clearer immuno-stained band has been demonstrated using mouse monoclonal antibody to fish Ig with salmonid rhabdoviruses IHNV and VHSV (Ristow *et al.*, 1993; Lorenzen *et al.*, 1993). More than 4 months after an outbreak of IHNV, most sera from surviving rainbow trout (9/28) gave a clear immuno-recognition band to viral protein M1 while some sera recognised protein bands G and M1. Ristow *et al.* (1993) also found that most surviving fish sera (82%) contained neutralising antibody as detected by plaque neutralisation tests. A few months after the outbreak of VHS, over 12 surviving trout sera from 55 serum samples gave positive plaque neutralisation tests but only 2 sera immuno-stained to protein G of VHSV in Western blot analysis (Lorenzen *et al.*, 1993). The possibility of the antibody response to rhabdovirus in EUS-recovered fish can protect or reduce the severity of EUS on subsequent years is still to be determined.

Chapter 7

7.1 Summary and Discussion

Two new cell lines were established from hybrid catfish. The head kidney-derived line was comprised of fibroblast-like cells and designated as HCK. The tail-derived line was epithelial in cell type and designated as HCT. Both cell lines grew best in Eagle's minimum essential medium (EMEM) containing 5-20% serum and had optimum growth temperatures ranging from 22°C to 28°C. HCK and HCT have been subcultured for 83 and 42 passages since initiation in 1991 and 1992 respectively. Both cell lines could be stored in liquid nitrogen for longer than 30 months but for only 3-6 months deep frozen at -70°C. The efficiency of plating of HCK at passage 60 was 32.3% and HCT at passage 22 was 16.8%. No adventitious viruses, bacteria, fungi or mycoplasmas could be detected in either cell line. HCT at passage 20 had a diploid chromosome number 2n = 55 while HCK at passage 19 and 63 might possess aneuploid chromosomes 2n = 63-64 and 64-66, respectively. Sixteen viruses have been tested for infectivity in the cell lines. Both lines were susceptible to infectious pancreatic necrosis virus (IPNV) serotypes Sp and Ab, golden shiner virus (GSV) and channel catfish reovirus (CRV). HCK was also susceptible to 2 reoviruses isolated from tench (*Tinca tinca*) and chub (*Leuciscus cephalus*). With reference to EUS-associated viruses selected for this study, both lines were susceptible to sand goby virus (SGV) but refractory to reovirus isolate T9231 and rhabdovirus isolates 20E, O2, SL11, BP, 19 and A4. Only HCK cell line was found susceptible to EUS-associated rhabdovirus isolate T9204. The results from the virus susceptibility study of the HCK cell line indicated that the earlier isolated EUS-

associated rhabdovirus strains were heterogeneous and might be divisible into 2 distinct rhabdovirus species as suggested by Lilley and Frerichs (1994). HCK showed a wider range of virus susceptibility than HCT and would be more suitable for virus isolation studies on EUS-diseased fishes and other fish diseases.

EUS is still a frightening disease for fish farmers as it has recurred every year in Thailand and in many other affected countries. In Thailand, the disease occurs during the cooler and dry season usually between December - February. During the present study, the 1993-1994 period of cool weather was very short (~2 weeks) and EUS outbreaks occurred about 1 week after the start of this period. The short period of cool weather did not encourage widespread EUS although the severity of the clinical condition in affected fish was much the same as in previous outbreaks. The disease may first occur in natural water or rice paddy field fish. If EUS is first observed in the natural water supply canal, the 2 immediate actions of cutting off the water running into the fish pond and applying lime are usually found to effectively prevent the spread of the disease into the pond. How these procedures work are not clearly understood but the first step may prevent a large number of fish pathogens getting access to the pond and the second step may kill some of the pathogens already present in the pond. Snakehead fish, three-spot gourami, snakeskin gourami, swamp eel, spiny eel and walking catfish were found sensitive to the 1993-1994 EUS outbreak while common carp, nile tilapia, barb, hybrid catfish, silver barb and climbing perch in diseased ponds or nearby ponds were not affected. Although the latter 2 species have been recorded as EUS-affected fish species (Tonguthai, 1985), they did not show any signs of disease during the observed outbreak.

A possible explanation may be that the cool season was too short or additional etiological factors may have been needed to induce the disease in these 2 fish species.

Nine virus strains were successfully recovered from EUS-diseased fishes in 1993-1994, 8 isolates from snakehead fish and 1 from three-spot gourami. All isolates belonged to a single virus family, Rhabdoviridae. Virus isolation was achieved when specimens were collected during the first 2 weeks of the outbreak. It was noted that tissue samples needed to be collected from moribund fish, processed and inoculated on to cell culture within a few days in order to obtain the best results. This is in general agreement with the virus isolation procedure described by Amos (1985). No virus was isolated from moribund fish examined 5 weeks after the start of the 1993-94 epizootic. BF-2 cells seemed to be the best cell line for virus isolation in this study followed by SSN-1 and HCK lines while no virus could be obtained using the EPC cell line. Interestingly, a repeat successful virus isolation was obtained from 5/6 moribund EUS-diseased snakehead fish which collected during early period (2-3 weeks) of the 1995-1996 outbreak from Phichit province, south region of the north of Thailand. 9 viruses were isolated from 11 tissue extracts and induced similar CPE in BF-2 cells while EPC and BB cells gave no CPE. One (AV9601/2) of them was identified as rhabdovirus and found to have serological properties similar to previous isolate T9204 from central Thailand but not to strain 20E from Myanmar using the neutralisation test.

A series of experimental infection studies was carried out using rhabdovirus isolate T9412 from snakehead fish from Bangkok. The results suggested that this new rhabdovirus isolate is pathogenic for fish as it caused death in snakehead fish fry. The

virus pathogen had very high virulence at low temperature with the LD_{50} = 2.16 $\log_{10}$ $TCID_{50}$/ml for snakehead fry within 11 days at 20°C. The moribund fry showed non-directional swirling movements. Virulence testing at the higher temperature of 28°C showed low or no mortality of snakehead fish fry. Silver barb fry, hybrid catfish fry, and young male guppy fish were found refractory to this rhabdovirus infection at both 20°C and 28°C. However, low mortality (~5%) in juvenile snakehead fish and minor clinical damage to the skin and muscle tissue in some fish were found in experimental infections. The persistence of rhabdovirus in bath- and i/p-infected fish held at 20°C showed that virus was rapidly eliminated from the fish and no virus was isolated on days 14 and 30 post-infection, respectively. Rhabdovirus seemed to persist longer in i/m-infected fish as 1 sample collected on day 30 post-infection still caused CPE in cell culture. The decrease in virus isolation with time in the experimental fish was directly related to the similar post-infection time for field virus isolation. How the viruses disappears from the host, whether fish surviving EUS and un-affected fish are virus carriers, whether there are other hosts apart from fish and how virus infection is cycled are questions which remain to be answered.

EUS induction in the laboratory was possible using both pathogenic rhabdovirus isolate T9412 and *Aphanomyces invaderis* strain RF-6. All 20 (100%) juvenile snakehead fish developed multiple EUS lesions by day 30 at 20°C when fish were first injected with virus followed by bath challenge with fungal spores. However, fish which received only L-15 medium by injection and were then bathed with fungal spores also developed EUS lesions although fewer (7/20) were affected. Some of these fish recovered and 15 fish appeared normal at the end of the 30 day experiment at 20°C. The lesions were

confirmed by the presence of mycotic granulomas in histological sections to be similar to the ulcerative changes in naturally EUS-diseased fish. The rhabdovirus injection alone induced only small haemorrhagic wounds at the i/m injection site in some fish held at 20°C, but most wounds healed by the end of the experiment and virus could be re-isolated from 20% (2/10) of surviving fish. A similar experiment conducted at 29°C failed to induce the EUS disease condition and no virus was recovered. EUS was also more readily transmitted from EUS-induced fish to other juvenile snakehead fish at 20°C than 28°C. During a 14 days EUS transmission trial at 20°C, snakehead fish which had survived 3 months after rhabdovirus infection were found very susceptible to EUS transmission. Temperature was found to be the most important factor regulating the interaction between rhabdovirus and *Aphanomyces* pathogens and the fish host in the laboratory. Similarly, in natural outbreaks of EUS, temperature is likely to be one of the most important environmental factors controlling the start and finish of epizootics in Thailand. This present study found that rhabdovirus caused skin damage in juvenile snakehead fish similar to previous experimental infections with EUS-associated birnavirus (Saitanu *et al.*, 1986) and UDRV strain BP and snakehead cell line retrovirus (Frerichs *et al.*, 1993). Based on the results of EUS induction experiments in this study, it would seem possible to induce the disease condition using a combination of pathogenic *Aphanomyces* and one of these viruses. However, rhabdoviruses may have a more significant role in the etiological complex than other EUS-associated viruses as they were the predominant isolates.

The new rhabdovirus isolates T9415, T9416 and T9429 had similar bullet- or bacillus-shaped morphology with mean dimensions ranging from 174-177 × 48-53 nm in ultra-

thin sections and a slightly larger size in negative stained virus pellet preparations with mean dimensions of 185-215 × 62-71 nm. The physico-chemical properties of the 3 virus isolates were similar to other fish rhabdoviruses as they were sensitive to heating at 56°C, pH 3 and lipid solvent but resistant to IUDR. The virus properties indicate that the rhabdoviruses possess a lipid containing envelope and a RNA genome. The new isolates had optimum growth temperature at 25-30°C and similar one-step growth cycles. The latent period of rhabdovirus multiplication lasted 2 h and new progeny was released at 2-4 h post-inoculation in FHM cells at 25°C. Growth reached a maximum at 17-22 h at which time the amount of virus in supernatant fluids was 8.5 $\log_{10}$ $TCID_{50}$/ml. All 3 isolates were able to infect and cause cytolysis in HCK, HCT, BF-2, FHM and SSN-1 but not in EPC, CHSE-214 and RTG-2 cell lines. Isolate T9416 showed some degree of dissimilarity to isolates T9415 and T9429 as it was the only tested rhabdovirus which infected BB and AS cell lines. All 3 new isolates are likely to belong to the same group as the earlier isolate T9204 as they were able to infect the HCK line.

From the mobility patterns of the structural proteins in SDS-PAGE, the 8 tested virus isolates could be separated into lyssavirus-like and vesiculovirus-like electrophoreotypes. The first group contained isolates T9415, T9416, T9429, SHRV and T9204 as they possessed 5 structural proteins comparable to proteins L, G, N, M1 and M2 of prototype *Lyssavirus*. The second group contained UDRV-SL11, -BP and -20E isolates as these viruses possessed structural proteins similar to protein L, G, N, (NS) and M of prototype *Vesiculovirus*. A possible NS protein might be located just below N protein in SDS-PAGE. Interestingly, the 3 new isolates, SHRV (Kasornchandra *et al.*, 1991) and T9204 (Lilley and Frerichs, 1994) of the lyssavirus group were isolated from EUS-

diseased fishes from central Thailand while viruses of the vesiculovirus group were isolated from Sri Lanka, northeast Thailand and Myanmar (Frerichs *et al.*, 1989b). Available information suggests that lyssavirus-like EUS-associated rhabdoviruses may have a specific geographical distribution linked to central Thailand whereas vesiculovirus-like EUS-associated rhabdoviruses seem to have a wider distribution. Antiserum to each rhabdovirus, except antiserum T9416, seemed to cross-neutralise only within the same electrophoreotype group which might represent 2 different serotypes. However, the variable degree of cross-neutralisation observed might be due to the low neutralising antibody titre of some antisera. Antiserum T9416 cross-neutralised all 7 tested rhabdoviruses of both serotypes. Antisera T9415, T9416, T9429 and T9204 could recognise all 5 structural proteins of homologous and heterologous viruses within the same electrophoreotype using immuno-staining on Western blot membranes. Antiserum 20E could immuno-stain only N, NS and M proteins of homologous and heterologous strains within the UDRV group of isolates. However, some degree of antigenic similarity was observed for the UDRV isolates when Western blot membranes were immuno-stained with antisera T9415, T9416, T9429 or T9204.

From the results of the virus characterisation study, rhabdovirus isolates T9415, T9429, SHRV and T9204 are structurally and serogically similar and the designation 'serotype Sh' is proposed for this group while isolates SL11, BP and 20E are grouped as proposed 'serotype Ud'. The serotype names are derived using the first 2 letters of SHRV and UDRV which were first used, respectively, by Kasornchandra *et al.* (1991) and Frerichs *et al.* (1989b). Virus members in each serotype are called rhabdovirus strains. The single isolate from three-spot gourami, strain T9416, could not be classified as a

memeber of either serotype Sh or Ud as the homologous antiserum was capable of neutralising viruses of both serotypes. The EUS-associated rhabdovirus isolated from snakehead fish from Bangkok was found to be pathogenic for fish. If other rhabdovirus strains are also proven to be fish pathogens, the term 'EUS-associated rhabdovirus' should be amended to the new term 'EUS rhabdovirus'. With reference to the proposal on Classification of Fish Viruses (Ahne, 1992), virus members of serotype Sh would be synonymous to *Lyssavirus ophicephalus* and virus members of serotype Ud are equivalent to *Vesiculovirus ophicephalus*. There is a need to produce an antiserum to strain SHRV and conduct a series of cross-neutralisation tests with new members in serotype Sh as this strain was the first isolated lyssavirus-like EUS-associated rhabdovirus, well characterised by Kasornchandra *et al.* (1991a; 1991b; 1992) and would represent the type strain of this serotype.

The unusual cross-neutralisation of the antiserum T9416 suggests that a mixture of serotype Sh and Ud viruses might be present in the T9416 isolate. Antiserum T9416 showed similar immuno-staining on N, NS, M and 32-33 kDa proteins to antiserum 20E but to a lesser degree. T9416 caused CPE in BB and AS cell lines while other lyssavirus-like EUS-associated rhabdovirus strains T9415, T9429, SHRV (Kasornchandra *et al.*, 1991) and T9204 (Lilley and Frerichs, 1994) were unlikely to induce CPE these 2 lines. Interestingly, the ability of CPE induction in BB and AS lines was more likely to be a characteristic of vesiculovirus-like EUS-associated rhabdovirus (Lilley and Frerichs, 1994; Frerichs *et al.*, 1989a). The eletronmicrograph of strain T9416 (Figure 6.1b) seemed to show 2 different sizes of rhabdovirus particles. If the mixed serotype postulation was true then the mixture of rhabdoviruses was most likely

to have originated from the diseased three-spot gourami as the virus had been passaged 3 times in the BF-2 line at AAHRI and twice more at the Institute of Aqauculture where the virus passage 5 was used for antiserum production in a rabbit. During the period of 5 passages, there was no experimental work or no handling of viruses of serotype Ud. If the postulation was false then the distinct characteristics of strain T9416 would indicate a third group of EUS-associated rhabdovirus. There is a need to establish the purity of strain T9416. As 3 virus families have been identified among EUS-diseased fishes, antiserum T9416 will be very useful to distinguish EUS-associated rhabdoviruses from EUS-associated birnaviruses and reoviruses.

The results of the present study suggest that rhabdovirus is one of a complex of etiological agents as it was easily isolated from moribund EUS-diseased fishes collected during the early period of the outbreak, one tested strain caused death at the fry stage and minor skin damage in the juvenile stage of snakehead fish and a combination of rhabdovirus and pathogenic *Aphanomyces* induced more severe EUS condition in snakehead fish juveniles at 20°C than a single infection with pathogenic fungus. Furthermore, the naturally EUS-recovered snakehead fish were found to have developed some antibody response against rhabdovirus strain T9429 as some recovered fish sera could reduce the infectivity of the virus for cell cultures and/or showed immuno-recognition to structural protein antigens of the rhabdovirus.

7.2 Further Investigations

The recent findings of this present study have supported the importance of rhabdovirus as one of the complex of etiological agents. However, the virus agent has a complicated relationship to the EUS outbreak and only some relationships were learned from the present study. Rhabdovirus was isolated for only a short period during the outbreak and its pathogenicity depended on age of fish, species of fish, routes of entry, and environmental temperature which were similar to other fish rhabdoviruses. Further relationships of viruses to EUS need to be examined in order to understand the role of viruses in the complex etiology. Some further studies are suggested as follows.

Testing the virulence of other EUS-associated rhabdoviruses:

- The fry stage of snakehead fish was found to be very sensitive to infection with the EUS-associated rhabdovirus strain T9412. It is recommended that the fry stage of snakehead fish and other related fish species be used to test the virulence of EUS-associated rhabdoviruses serotype Ud, other strains of serotype Sh and the isolate T9416 at ~20°C. If all tested viruses cause death in fish fry then '-associated' should be deleted from the descriptive terminology. The virulence tests should also apply to EUS-associated birnaviruses and reovirus.
- The juvenile stage of snakehead fish was found less sensitive with slower developing clinical signs than fry following rhabdovirus infection. It would be instructive to study the pathogenesis of rhabdovirus infection by applying immuno-detection techniques such as immunofluorescence or immunohistochemistry to elucidate routes of virus entry and localisation of virus in the fish body including target organs.

Optimising tissue preparations for virus isolation:

- The results of this study showed that moribund fish specimens are essential for virus isolation and tissue preparation and inoculation should be done within a few days of collection. The problems experienced in this study were that the EUS-diseased fish often died during transportation and it usually takes some days to complete virus isolation process. A series of experiments needs to be conducted to define optimal conditions for collecting and transporting fresh specimens and retaining tissue extracts which will be a very useful guideline for collection and transportation of EUS-diseased specimens from different geographical areas or countries.

Conducting further virus isolation studies:

- Rhabdoviruses were isolated only during the early period of EUS. To further understand the source of the viruses in nature and when fish become infected, a series of tissue samples for virus isolations should be collected from a range of fish and invertebrate species in the affected areas before, during and after an outbreak of disease.
- EUS-diseased fish may be infected by a number of viruses. To isolate different viruses, cell lines must be carefully selected. For example, the HCK line is sensitive to rhabdovirus serotypes Sh and birnavirus (SGV) but refractory to rhabdovirus serotype Ud and reovirus T9231. However, modifications may be applied to tissue preparations for isolation of different viruses in the extract. The extract can be treated with a lipid solvent or antiserum against known viruses before inoculation on to cell culture as has been successfully used to isolate IPNV and VHSV from rainbow

trout (Jørgensen, 1982a). Plaque purification assays may also be used for examining tissue extracts but this needs an appropriate cell line, a suitable overlay medium and a skilled virologist to interpret differences in plaque morphology on the cell monolayers. A recent plaque assay trial indicated that there were no differences in plaque morphology in SSN-1 cells induced by strains UDRV-SL11 and UDRV-19 of serotype Ud and strain T9204 of serotype Sh (Nurtjahya, 1995). The assay might not differentiate EUS-associated rhabdoviruses but it may indicate differences in plaque morphology caused by EUS-associated birnavirus or reovirus. Wolf and Quimby (1973) demonstrated that different viruses could induce different plaque morphology which could be used as presumptive identification of known viruses from the diseased fishes.

Testing for a possible mixture of rhabdovirus serotype Sh and Ud in the strain T9416:

- Antiserum T9416 had the ability to cross-neutralise rhabdovirus serotype Sh and Ud which could indicate a mixture of viruses of both serotypes in the antigen preparation used for rabbit immunisation. To separate out virus members of serotype Sh, strain T9416 should be incubated with antiserum 20E. To separate only viruses of serotype Ud, the suspected mixture of viruses should be reacted with antiserum T9415. The trials may be performed using virus neutralisation or plaque reduction tests. If strain T9416 is found to be a single species of rhabdovirus then it would be very interesting to determine which viral proteins give a neutralising antibody to viruses of both serotypes. Therefore, mono-specific polyclonal antibody to strain T9416 is needed for cross-neutralisation tests.

Detecting antibody response in EUS-recovered fish:

- The preliminary results reported here have shown that EUS-recovered fish developed some degree of antibody response to rhabdovirus antigen. It would be useful to conduct virus neutralisation, ELISA or Western blot analysis with serum from EUS-recovered fish from future outbreaks as the EUS-diseased fishes may not be easily collected in the affected fish culture pond (Thai fish farmers usually do not want the fish to be disturbed or frightened). The latter two tests may need monoclonal anti-fish IgM in order to enhance test sensitivity. A rhabdovirus strain of each serotype or other related viruses should be used in the tests. The results may help identify which group of viruses might be involved in the epizootics.

Developing immuno-diagnostic or molecular diagnostic tests:

- Fresh diseased fish specimens for virus isolation are usually difficult to obtain from remote EUS-affected areas or other countries. There is a need to develop immuno-diagnostic or molecular diagnostic tests as alternatives to virus isolation. The fresh specimens may be frozen or fixed in formalin or alcohol which can be examined later by enzyme-linked immunosorbant assay, immunofluorescence, immunoperoxidase, radioimmune assay, dot immunoblot assay, DNA probes or PCR technique. The PCR is a very sensitive and advanced technique for detecting virus genome in fresh or formalin-fixed and paraffin-embedded fish tissue (Chiou *et al.*, 1995).

References

Adams, R.L.P. (1980). *Cell Culture for Biochemists: Laboratory Techniques in Biochemistry and Molecular Biology,* Vol. 8. Elsevier/North-Holland Biomedical Press, The Netherlands.

Ahne, W. (1975). A rhabdovirus isolated from grass carp (*Ctenopharyngodon idella* Val.). *Arch. Virol.* 48-181-185.

Ahne, W. (1978). Uptake and multiplication of spring viremia of carp virus in carp, *Cyprinus carpio* L. *J. Fish Dis.* 1:265-268.

Ahne, W. (1980). Rhabdovirus carpio-infektion beim karpfen (*Cyprinus carpio*): untersuchungen über peaktionen des wirtsorganismus. *Fortschr. Veterinärmed.* 30:180-183.

Ahne, W. (1981). Serological techniques currently used in fish virology. *Devel. Biol. Stand.* 49:3-27.

Ahne, W. (1986). The influence of environmental temperature and infection route on the immune response of carp (*Cyprinus carpio*) to spring viremia of carp virus (SVCV). *Veterinary Immunology and Immunopathology.* 12:383-386.

Ahne, W. (1992). Classification and nomenclature of fish viruses. In *Salmonid Diseases: Proceeding of the OJI International Symposium on Salmonid Diseases* (Kimura, T., ed.) pp. 124-136. Hokkaido University Press, Japan.

Ahne, W. & Hölbl, O. (1988). Some properties of reovirus isolated from tench (*Tinca tinca*) and chub (*Leuciscus cephalus*). In *Viruses of Lower Vertebrates* (Ahne, W. & Kurstak, E., eds.) pp.250-256. Springer-Verlag, Berlin.

Ahne, W., Jørgensen, P.E.V., Olesen, N.J. & Wattanavijarn, W (1988). Serological examination of a rhabdovirus isolated from snakehead fish (*Ophicephalus striatus*) in Thailand with ulcerative syndrome. *J. appl. Ichthyol.* 4:194-196.

Almeida-Toledo, L.F., Viegas-Péquignot, E., Foresti, F., Toledo-Filho, S.A. & Dutrillaux, B. (1988). BrdU replication patterns demonstrating chromosome homoeologies in two fish species, genus *Eigenmannia*. *Cytogenet Cell Genet.* 48:117-120.

Alvarez, M.C., Otis, J., Amores, A. & Guise, K. (1991). Short-term cell culture technique for obtaining chromosomes in marine and freshwater fish. *J. Fish Biol.* 39:817-824.

Amemiya, C.T., Bickham, I.W. & Gold, R. (1984). A cell culture technique for chromosome preparation in cyprinid fishes. *Copeia* 1984, 232-235.

Amend, D.F. (1970). Control of infectious hematopoietic necrosis virus disease by elevating the water temperture. *J. Fish. Res. Board Can.* 27:265-270.

Amos, K.M. (ed.) (1985). *Fish Health Blue Book. Procedures for the detection and identification of certain fish pathogens,* 3rd edn. Fish Health Section, American Fisheries Society, Corvallis, Oregon.

Anderson, C.D. & Roberts, R.J. (1975). A comparison of the effects of temperature on wound healing in a tropical and temperate teleost. *J. Fish Biol.* 7:173-182.

Andral, R., Hurard, C., Elziere-Papayanni, P. & Vivarès, C.P. (1990). Establishment and characterization of a rainbow trout kidney cell-line, RTK Montpellier. In *Pathology in Marine Science* (Perkins, F.O. & Cheng, T.C., eds.) pp. 33-42. Academic Press, New York.

Arkush, K.D., Bovo, G., de Kinkelin, P., Winton, J.R., Wingfield, W.H. & Hedrick, R.P. (1989). Biochemical and antigenic properties of the first isolates of infectious hematopoietic necrosis virus from salmonid fish in Europe. *J. Aquat. Animal Health.* 1:148-153.

Babich, H. & Borenfreund, E. (1991). Cytotoxicity and genotoxicity assays with cultured fish cells: a review. *Toxic. in Vitro.* 5(1):91-100.

Balasuriya, K.S.W., Kulathilake, M. & Subasinghe, R.P. (1990). Preliminary investigations into the experimental transmission of ulcerative diseases syndrome in fish in Sri Lanka. In *The Second Fisheries Forum* (Hirano, R. & Hanyo, I., eds.) pp. 659-662. Asian Fisheries Society, Manila, Philippines.

Barua, G. (1994). The status of epizootic ulcerative syndrome of fish of Bangladesh. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp. 13-20. Aquatic Animal Health Research Institute, Bangkok.

Baudouy, A.M., Danton, M. & Merle, G. (1980). Experimental infection of susceptible carp fingerlings with spring viremia of carp virus under wintering environmental conditions. In *Fish Diseases. Third COPRAQ Session* (Ahne, W., ed.) pp. 23-27. Springer-Verlag, Berlin.

Bazzola, J.J. & Russell, L.D. (1992). *Electron Microscopy Principles and Techniques for Biologists.* Jones and Bartlett Publishers, Boston.

Bjare, U. (1992). Serum-free cell culture. *Pharmac. Ther.* 53:355-374.

Björklund, H.V., Olesen, N.J. & Jørgensen, P.E.V. (1994). Biophysical and serological characterization of rhabdovirus 903/87 isolated from European lake trout *Salmo trutta lacustris*. *Dis. aquat. Org.* 19:21-26.

Bols, N.C. (1991). Biotechnology and aquaculture: the role of cell cultures. *Biotech. Adv.* 9:31-49.

Bols, N.C. & Lee, L.E.J. (1991). Technology and uses of cell cultures from the tissues and organs of bony fish. *Cytotechnology.* 6:163-187.

Bondad-Reantaso, M.G., Catap, E.S., Paclibare, J.O., Lumanlan-Mayo, S.C. & Callinan, R.B. (1994). Environmental factors associated with EUS occurrence in freshwater and estuarine areas in Luzon. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp 76-81. Aquatic Animal Health Research Institute, Bangkok.

Boonyaratpalin, S. (1989a). Bacterial pathogens involved in the epizootic ulcerative syndrome of fish in southeast Asia. *J. Aquat. Animal Health.* 1:272-276.

Boonyaratpalin, S. (1989b). *A report on epizootic ulcerative sysndrome of fish in India.* Paper code NACA/WP/89/80, Network of Aquaculture Centres in Asia, Bangkok.

Boonyaratpalin, S., Kasornchandra, J. & Roenviroj, S. (1983). *Ulcer disease in snakehead (Ophicephalus striatus* Bloch*).* Technical Report no. 28, National Inland Fisheries Institute, Bangkok. (English abstract)

Bootland, L.M., Lorz, H.V., Rohovec, J.S. & Leong, J.C. (1994). Experimental infection of brook trout with infectious hematopoietic necrosis virus type 1 and 2. *J. Aquat. Animal Health.* 6:144-148.

Bootsma, R., de Kinkelin, P. & LeBerre, M. (1975). Transmission experiments with pike fry (*Esox lucius* L.) rhabdovirus. *J. Fish Biol.* 7:269-276.

Bovo, G., Olesen, N.J., Jørgensen, P.E.V., Ahne, W. & Winton, J.R. (1995). Characterization of a rhabdovirus isolated from carpione *Salmo trutta carpio* in Italy. *Dis. aquat. Org.* 21:115-122.

Bowser, P.R. & Plumb J.A. (1980). Growth rates of a new cell line from channel catfish ovary and channel catfish virus replication at different temperatures. *Can. J. Fish. Aquat. Sci.* 37:871-873.

Brown, F. (1987). The family Rhabdoviridae: general description and taxonomy. In *The Rhabdoviruses* (Wagner, R.R., ed.) pp. 1-8. Plenum Press, New York.

Bullock, A.M., Marks, R. & Roberts, R.J. (1978). The cell kenetics of teleost fish epidermis: epidermal mitotic activity in relation to wound healing at varying temperatures in plaice (*Pleuronectes platessa*). *J. Zool., Lond.* 185:197-204.

Burleson, F.G., Chambers, T.M. & Wiedbrauk, D.L. (1992). *Virology: a Laboratory Manual*. Academic Press, London.

Callinan, R.B. (1994). Red spot disease – EUS in Australia. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp 82-88. Aquatic Animal Health Research Institute, Bangkok.

Callinan, R.B. & Keep, J.A. (1989). Bacteriology and parasitology of red spot disease in sea mullet, *Mugil cephelus* L., from eastern Australia. *J. Fish Dis.* 12:349-356.

Callinan, R.B., Paclibare, J.O., Bondad-Reantaso, M.G., Lumanlan-Mayo, S.C., Fraser, G.C. & Sammut, J. (1995). EUS outbreaks in estuarine fish in Australia and the Philippines: associations with acid sulphate soils, rainfall and *Aphanomyces*. In *Diseases in Asian Aquaculture II* (Shariff, M., Arthur, J.R. & Subasinghe, R.P., eds.) pp. 291-298. Fish Health Section, Asian Fisheries Society, Manila.

Castric, J. & Chastel, C. (1980). Isolation and characterization attempts of three viruses from European eel, *Anguilla anguilla*: preliminary results. *Ann. Virol. (Inst. Pasteur).* 131E:435-448.

Castric, J. & de Kinkelin, P. (1984). Experimental study of the susceptibility of two marine fish species, sea bass (*Dicentrachus labrax*) and turbot (*Scophthalmus maximus*), to viral hemorrhagic septicaemia. *Aquaculture* 41:203-212.

Castric, J., Rasschaert, D. & Bernard, J. (1984). Evidence of lyssavirus among rhabdovirus isolates from the European eel *Anguilla anguilla*. *Ann. Virol. (Inst. Pasteur).* 135E:35-55.

Chen, S.N. & Kou, G.H. (1988). Establishment, characterization and application of 14 cell lines from warm-water fish. In *Invertebrate and Fish Tissue Culture* (Kuroda, Y., Kurstak, E. & Maramorosch, K., eds.) pp. 195-198. Springer-Verlag, New York.

Cheng, Li-Lin., Bowser, P.R. & Spitsbergen, J.M. (1993). Development of cell cultures derived from lake trout liver and kidney in a hormone-supplemented, serum-reduced medium. *J. Aquat. Animal Health.* 5:119-126.

Chinabut, S. (1995). Epizootic ulcerative syndrome: the current state of knowledge. In *Diseases in Asain Aquaculture II* (Shariff, M., Arthur, J.R. & Subasinghe, R.P., eds.) pp. 285-290. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Chinabut, S. & Limsuwan, C. (1983). Histopathological changes in some freshwater fishes found during the disease outbreak: 1982-1983. *Thai Fisheries Gazette.* 36(3):281-289. (English Abstract)

Chinabut, S., Roberts, R.J., Willoughby, G.R. & Pearson, M.D. (1995). Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. *J. Fish Dis.* 18:41-47.

Chiou, P.P., Drolet, B.S. & Leong, J.C. (1995). Polymerase chain amplification of infectious hematopoietic necrosis virus RNA extracted from fixed and embedded fish tissue. *J. Aquat. Animal Health.* 7:9-15.

Clerx, J.P.M. & Horzinek, M. (1978). Comparative protein analysis of non-salmonid fish rhabdoviruses. *J. gen. Virol.* 40:287-295.

Costa, H.H. & Wijeyaratne, M.J.S. (1989). Epidemiology of the epizootic ulcerative syndrome occurring for the first time among fish in Sri Lanka. *J. appl. Ichthyol.* 1:48-52.

Cowin, P. & Garrod, D.R. (1983). Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. *Nature Lond.* 302:148-150.

Cowin, P., Mattey, D.L. & Garrod, D.R. (1984). Distribution of desmosomes components in the tissue of vertebrates studied by fluorescent antibody staining. *J. Cell Sci.* 66:119-132.

Cox, J.H., Holloway, B.P. & Obijeski, J.F. (1977). Rabies virus glycoprotein. II. biological and serological characterization. *Infection and Immunity* 16:754-759.

Cruz-Lacierda, E.R. & Shariff, M. (1995). Experimental transmission of epizootic ulcerative syndrome (EUS) in snakehead, *Ophicephalus striatus.* In *Diseases in Asian Aquaculture II* (Shariff, M., Arthur, J.R. & Subasinghe, R.P., eds.) pp. 327-336. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Cruz-Lacierda, E.R. & Shariff, M. (1996). Histopathology of epizootic ulcerative

syndrome-positive snakehead, *Ophicephalus striatus*. *Annual Meeting of the world Aquaculture Society, January 29 - February 2, 1996*. Department of Fisheries and the Chulabhorn Research Institute, Bangkok. (Abstract p. 94)

Darlington, R.W., Trafford, R. & Wolf, K. (1972). Fish rhabdoviruses: morphology and ultrastructure of North American salmonid isolates. *Archiv fur die gesamate virusforschung*. 39:257-264.

Das, M.K. (1994). Outbreak of the fish disease, epizootic ulcerative syndrome in India: an overview. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp 21-38. Aquatic Animal Health Research Institute, Bangkok.

de Kinkelin, P. & LeBerre, M. (1979). Mass virus production in fish cell system. *Develop. biol. Stand.* 42:99-104.

de Kinkelin, P., Galimard, B. & Bootsma, R. (1973). Isolation and identification of the causative agent of "red-disease" of pike (*Esox lucius* L.1766). *Nature Lond.* 241:465-467.

de Kinkelin, P., LeBerre, M. & Lenoir, G. (1974). Rhabdovirus des poissons. I. properties *in vitro* de virus de la maladie rouge de l'alevin de brochet. *Annals of Microbiology (Paris)*. 125A:93-111.

Delany, M.E. & Bloom, S.E. (1984). Replication banding patterns in the chromosomes of the rainbow trout. *The Journal of Heredity*. 75:431-434.

Dietzschold, B., Schneider, L.G. & Cox, S.H. (1974). Serological characterization of the three major proteins of vesicular stomatitis virus. *J. Virol.* 14:1-7.

Dorson, M., Torchy, C., Chilmonczyk, S., de Kinkelin, P. & Michel, C. (1984). A rhabdovirus pathogenic for pearch, *Perca fluviatilis* L.: isolation and preliminary study. *J. Fish Dis.* 7:241-245.

Dubois-Dalcq, M., Holmes, K.V., Rentier, B. & Kingsbury, D.W. (eds.) (1984). Assembly of rhabdoviridae. In *Assembly of enveloped RNA viruses* pp. 23-43. Springer, New York.

Egusa, S., & Masuda, N. (1971). A new fungal disease of *Plecoglossus altivelis*. *Fish Pathology*. 6: 41-46.

Engelking, H.M. & Leong, J.C. (1989). The glycoprotein of infectious hematopoietic necrosis virus elicits neutalizing antibody and protective responses. *Virus Research*. 13:213-230.

Engelking, H.M., Harry, J.B. & Leong, J.C. (1991). Comparison of representative strains of infectious hematopoietic necrosis virus by serological neutralization and cross-protection assay. *Applied and Environmental Microbiology*. 57:1372-1378.

Faisal, M., Hoegerman, S.F., Rutan, B.J. & Sami, S. (1992). Chromosome analysis using fish leukocytes and cultured cells. In *Techniques in Fish Immunology* (Anderson, D.P., Kaattari, S.L. & Rowley, A.F., eds.) pp. 185-195. SOS Publications, Fair Haven, Newjercy.

FAO. (1986). *Report of the Expert Consultation on Ulcerative Fish Diseases in the Asia-Pacific Region*. TCP/RAS/4508 Project, FAO, Bangkok.

Fernandez, R.D., Yoshimizu, M. Kimura, T., Ezura, Y., Inouye, K. & Takami, I. (1993a). Characterization of three continuous cell line from marine fish. *J. Aquat. Animal Health*. 5:127-136.

Fernandez, R.D., Yoshimizu, M., Kimura, T. & Ezura, Y. (1993b). Establishment and characterization of seven continuous cell lines from freshwater fish. *J. Aquat. Animal Health*. 5:137-147.

Fijan, N., Petrinec, Z., Sulimanovic, D. & Zwillenberg, L.O. (1971). Isolation of the causative agent from the acute form of infectious dropsy of carp. *Veterinarski Arhiv*. 41:125-138.

Fijan, N., Sulimanovic, D., Bearyotti, M., Muzinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J,F. & de Kinkelin, P. (1983). Some properties of the *Epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio*. *Ann. Virol. (Inst. Pasteur)*. 134E:207-220.

Fijan, N., Wellborn, T.L. Jr. & Naftel, J.P. (1970). *An acute viral disease of channel catfish*. Technical Paper No. 43, U.S. Fish Wildl. Serv., USA.

Frantsi, C. & Savan, M. (1971). Infectious pancreatic necrosis virus: temperature and age factors in mortality. *J. Wildl. Dis*. 7:249-255.

Fraser, G.C., Callinan, R.B. & Calder, L.M. (1992). *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis*. 15:173-181.

Frederick, A.M. & Kingsbury, D.W. (1990). Virus taxonony. In *Virology*, 2nd edn. (Fields, B.N., Knipe, D.M., Chanock, R.M., Hirsch, M.S., Melnick, J.L., Monath, T.P. & Roizman, B., eds.). Raven Press, New York.

Frerichs, G.N. (1989). Rhabdoviruses of fishes. In *Viruses of Lower Vertebrates* (Ahne, W. & Kurstake, E., eds.) pp. 317-332. Springer-Verlag, Berlin.

Frerichs, G.N. (1995). Viruses associated with the epizootic ulcerative syndrome (EUS) of fish in south-east Asia. *Vet. Res.* 26: 449-454.

Frerichs, G.N., Hill, B.J. & Way, K. (1989a). Ulcerative disease rhabdovirus: cell-line susceptibility and serological comparison with other fish rhabdoviruses. *J. Fish Dis.* 12:51-56.

Frerichs, G.N., Millar, S.D. & Alexander, M. (1989b). Rhabdovirus infection of ulcerated fish in South-East Asia. In *Viruses of Lower Vertebrates* (Ahne, W. & Kurstak, E., eds.) pp. 396-410. Springer-Verlag, Berlin.

Frerichs, G.N., Millar, S.D. & Chinabut, C. (1993). Clinical response of snakeheads (*Ophicephalus striatus*) to experimental infection with snakehead fish rhabdovirus and snakehead cell line retrovirus. *Aquaculture*. 116: 297-301.

Frerichs, G.N., Millar, S.D. & Roberts, R.J. (1986). Ulcerative rhabdovirus in fish in South-East Asia. *Nature Lond.* 322: 216.

Frerichs, G.N., Morgan, D., Hart, D., Skerrow, C., Roberts, R.J. & Onions, D.E. (1991). Spontaneously productive C-type retrovirus infection of fish cell lines. *J. gen. Virol.* 72:2537-2539.

Freshney, R.I. (1987). *Culture of Animal Cells: a Manual of Basic Technique*, 2nd edn. Alan R. Liss, New York.

Fryer, J.L. & Lannan, C.N. (1994). Three decades of fish cell culture: a current listing of cell lines derived from fishes. *J. Tissue Culture Methods*. 16:87-94.

Fryer, J.L., McCain, B.B. & Leong, J.C. (1981). A cell line derived from rainbow trout (*Salmo gairdneri*) hepatoma. *Fish Pathology*. 15:193-200.

Garrod, D.R. (1986). Desmosomes, cell adhesion molecules and the adhesive properties of cell in tissue. *J. Cell Sci. Suppl.* 4:221-237.

Gravell, M. & Malsberger, R.G. (1965). A permanent cell line from the fathead minnow (*Pimephales promelas*). *Ann. N.Y. Acad. Sci.* 126:555-565.

Griffith, O.M. (1986). *Techniques of Preparative, Zonal and Continuous Flow Ultracenrifugation*, 5th edn. Beckman Instruments Inc., California.

Habashi, Y., Schlotfeldt, H.-J. & Frost, J.W. (1975). Production of antisera against the virus of viral haemorrhagic septicaemia (VHS) of rainbow trout. *Zbl. Vet. Med. B.* 22:666-672.

Hames, B.D. & Rickwood, D. (eds.) (1990). *Gel Electrophoresis of Proteins: a Practical Approach,* 2nd edn. Oxford University Press, Oxford, UK.

Harmon, S.A. & Summers, D.F. (1982). Characterization of monospecific antisera against all five vesicular stomatitis virus-specific proteins: anti-L and anti-NS inhibit transcription *in vitro*. *Virology*. 120:194-204.

Hatai, K. (1980). *Studies on Pathogenic Agents of Saprolegniasis in Fresh Water Fishes.* Special Report No. 8, Nagasaki Pref. Inst. Fish. 95 p. (English Abstract)

Hay, R.J. (1985). *ATCC Quality Control Methods for Cell Lines.* American Type Culture Collection, Maryland.

Hayat, M.A. (1989). *Principles and Techniques of Electron Microscopy: Biological Applications*, 3rd edn. Macmillan Press, London.

Hedrick, R.P., Eaton, W.D., Fryer, J.L., Groberg, W.G. Jr. & Boonyaratpalin, S. (1986). Characteristics of a birnavirus isolated from cultured sand goby, *Oxyeleotris marmoratus*. *Dis. aquat. Org.* 1:219-225.

Hedrick, R.P., Rosemark, R., Aronstein, D., Winton, J.R., McDowell, T. & Amend, D.F. (1984). Characteristics of a new reovirus from channel catfish (*Ictalurus punctatus*). *J. gen. Virol.* 65:1527-1534.

Hetrick, F.M. (1989). Fish viruses. In *Methods for the Microbiological Examination of Fish and Shellfish* (Austin, B. &Austin, D.A., eds.) pp. 216-239. Ellis Horwood Ltd., Chichester, UK

Hill, B.J., Underwood, B.O., Smale, C.J. & Brown, F. (1975). Physico-chemical and serological characterization of five rhabdoviruses infecting fish. *J. gen. Virol.* 27:369-378.

Hill, B.J., Williams, R.F., Smale, C.J., Underwood, B.O. & Brown, F. (1980). Physico-chemical and serological characterization of two rhabdoviruses isolated from eels. *Intervirology* 14:208-212.

Hill,B.J., Williams, R.F. & Finlay, J. (1981). Preparation of antisera against fish virus disease agents. *Develop. Biol. Stand.* 49:209-218.

Hossain, M.S., Alam, M. & Mazid, M.A. (1992). Survey on fish disease "Epizootic ulcerative syndrome" in Chandpur district. *Bangladesh Journal of Training and Development.* 5(1):55-61.

Hsu, Y.L. & Leong, J.C. (1985). A comparison of detection methods for infectious hematopoietic necrosis virus. *J. Fish Dis.* 8:1-12.

Hsu, Y.L., Engelking, H.M. & Leong, J.C. (1986). Occurrence of different types of infectious hematopoietic necrosis virus in fish. *Applied and Environmental Microbiology.* 52:1353-1361.

Huang, C., Chien, M.S., Landolt, M. & Winton, J.R. (1994). Characterization of the infectious hematopoietic necrosis virus glycoprotein using neutralizing monoclonal antibodies. *Dis. aquat. Org.* 18:29-35.

Humphrey, J.D., & Langdon, J.S. (1986). *Ulcerative disease in Northern Territory fish.* Internal Report, Australian Fish Health Reference Laboratory, Benalla, Victoria, Australia, 13 p.

Jensen, M. (1963). Preparation of fish tissue cultures for virus research. *Bull. Off. Int. Epizoot.* 59:131-134.

Jensen, M. (1965). Research on the virus of Egtved disease. *Ann. N.Y. Acad. Sci.* 126:422-426.

Jensen, N.J., Bloch, B. & Larsen, J.L. (1979). The ulcus-syndrome in cod (*Gadus morhua*), III: a preliminary virological report. *Nord. Vet. Med.* 31:436-442.

Jørgensen, P.E.V., Olesen, N.J., Ahne, W. & Lorenzen, N. (1989). SVCV and PFR virus. Serological examination of 22 isolates indicates close relationship between the two fish rhabdoviruses. In *Viruses of Lower Vertebrates* (Ahne, W. & Kurstak, E., eds.) pp. 349-366. Springer-Verlag, Berlin.

Jørgensen, P.E.V. (1972). Egtved virus: antigenic variation in 76 virus isolates examined in neutralization tests and by means of the fluorescent antibody technique. *Symposium of the Zoological Society of London.* 30:333-340.

Jørgensen, P.E.V. (1982a). Egtved virus: occurrence of inapparent infections with virulent virus in free-living rainbow trout, *Salmo gairdneri* Richardson, at low temperature. *J. Fish Dis.* 5:251-255.

Jørgensen, P.E.V. (1982b). Egtved virus: temperature-dependent immune response of trout to infection with low-virulence virus. *J. Fish Dis.* 5:47-55.

Jørgensen, P.E.V. & Olesen, N.J. (1987). Cod ulcus syndrome rhabdovirus is indistinguishble from the Egtved (VHS) virus. *Bull. Eur. Fish Pathol.* 7(3):73-74.

Jørgensen, P.E.V., Olesen, N.J., Ahne, W., Wahli, T. & Meier, W. (1993). Isolation of a previously undescribed rhabdovirus from pike *Esox lucius*. *Dis. aquat. Org.* 16:171-179.

Kanchanopas, P. (1984). *Effect of paraquat on snake-head fish*. M.Sc. Dissertation, Faculty of Fisheries, Kasetsart University, Bangkok.

Kang, C.Y. & Prevec, L. (1970). Proteins of vesicular stomatitis virus: II Immunological comparisons of viral antigens. *J. Virol.* 6:20-27.

Kärber, G. (1931). Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. *Arch. Exp. Pathol. Pharm.* 162:480.

Karnovsky, M.J. (1965). A formaldehyde-glutaradehyde fixative of high osmolarity for use in electron microscopy. *Journal of Cell Biology*. 27:137A-138A.

Karunasagar, I., Sugumar, G. & Karunasagar, I. (1995). Virulence characters of *Aeromonas* spp. isolated from EUS-affected fish. In *Diseases in Asian Aquaculture II* (Shariff, M., Arthur, J.R. & Subasinghe, R.P., eds.) pp. 307-314. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Kasornchanadra, J., Boonyaratpalin, S. & Saduadee, J. (1988). *Fish cell line initiated from snakehead fish (Ophicephalus striatus, Bloch) and its characters.* Technical Paper No. 90, National Inland Fisheries Institute, Department of Fisheries, Bangkok.

Kasornchandra, J., Caswell-Reno, P. & Lannan, C.N. (1992a). Development and characterization of monoclonal antibodies against the snakehead rhabdovirus. *J. Aquat. Animal Health.* 4:286-291.

Kasornchandra, J., Engelking, H.M., Lannan, C.N., Rohovec, J.S. & Fryer, J.L. (1992b). Characterization of the rhabdovirus from snakehead fish *Ophicephalus striatus*. *Dist. aquat. Org.* 13:89-94.

Kasornchandra, J., Lannan, C.N., Rohovec, J.R. & Fryer, J.L. (1991). Characterization of a rhabdovirus isolated from the snakehead fish (*Ophicephalus striatus*). In *Proceedings of the Second International Symposium on Viruses of Lower Vertebrates* (Fryer, J.L., ed.) pp. 175-182. Oregon State University, Corvallis, Oregon.

Kelley, J.M., Emerson & Wagner, R.R. (1972). The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. *J. Virol.*

10:1231-1235.

Kimura, T., Yoshimizu, M. & Gorie, S. (1986). A new rhabdovirus isolated in Japan from cultured hirame (Japanese flounder, *Paralichthys olivaceus*) and ayu (*Plecoglossus altivelis*). *Dis. aquat. Org.* 1:209-217.

Kirchhoff, H., Rosengarten, R. & Chercheletzi, C. (1983). Isolation of mycoplasmas from a tench (*Tinca tinca* L.). *Yale Journal of Biology and Medicine*. 56:841-842.

Koski, P., Hill, B.J., Way, K., Neuvonen, E. & Rintämaki, P. (1992). A rhabdovirus isolated from brown trout (*Salmo trutta lacustris* L.) with lesions in parenchymatous organs. *Bull. Eur. Ass. Fish Pathol.* 12:177-180.

Lannan, C.N., Winton, J.R. & Fryer, J.L. (1984). Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro*. 20(9):671-676.

Lawanyawut, K., Penman, D.J., McAndrew, B.J. & Roongratri, N. (1992). Preliminary study on karyotyping of the hybrid catfish pla duk big-oui (male *Clarias gariepinus* × female *C. macrocephalus*). In *AADCP Workshop Proceedings: Genetics in Aquaculture and Fisheries management* (Penman, D.J., Roongratri, N. & McAndrew, B., eds.) pp. 141-142. University of Stirling, Scotland.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature Lond.* 227:680-685.

Lechner, J.F. (1985). Inhibition by serum. In *Uses and Standardization of Vertebrate Cell Cultures* (Patterson, M.K.Jr. ed.) pp. 80-82. Tissue Culture Association, Gaithersburg, Maryland.

Lenoir, G. & de Kinkelin, P. (1975). Fish rhabdoviruses: comparative study of protein structure. *J. Virol.* 16:259-262.

Leong, J.C. (1995). Molecular biological tools to detect fish pathogens. *J. Fish Biol.* 47(Suppl. A):61-75.

Lidgerding, B.C. (1981). Cell lines used for the production of viral fish disease agents. *Develop. biol. Stand.* 49:233-241.

Lilley, J.H. & Frerichs, G.N. (1994). Comparison of rhabdoviruses associated with epizootic ulcerative syndrome (EUS) with respect to their structural proteins, cytopathology and serology. *J. Fish Dis.* 17:513-522.

Lilley, J.H., Phillips, M.J. & Tonguthai, K. (1992). *A Review of Epizootic Ulcerative*

Syndrome (EUS) in Asia. Aquatic Animal Health Research Institute and Network of Aquaculture Centres in Asia, Bangkok.

Llobrera, A.T. & Gacutan, R.Q. (1987). *Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Philippines. *Aquaculture*. 67:273-278.

Lorenzen, N., Olesen, N.J. & Jørgensen, P.E.V. (1988). Production and characterization of monoclonal antibodies to four Egtved virus structural proteins. *Dis. aquat. Org.* 4:35-42.

Lorenzen, N., Olesen, N.J., Jørgensen, P.E.V., Etzerodt, M., Holtet, T.L. & Thogersen, H.C. (1993). Molecular cloning and expression in *Escherichia coli* of the glycoprotein gene of VHS virus and immunization of rainbow trout with the recombinant protein. *J. gen. Virol.* 74:623-630.

Lu, Y., Lannan, C.N., Rohovec, J.S. & Fryer, J.L. (1990). Fish cell lines: establishment and characterization of three new cell lines from grass carp (*Ctenopharyngodon idella*). *In Vitro Cell. Dev. Biol.* 26:275-279.

Matthews, R.E.F. (1982). Classification and nomenclature of viruses: fourth report of the international committee on taxonomy of viruses. *Intervirology*. 17:1-199.

McAllister, P.E. & Wagner, R.R. (1975). Structural proteins of two salmonid rhabdoviruses. *J. Virol.* 15(4):733-738.

McAllister, P.E., Fryer, J.L. & Pilcher, K.S. (1974). Further characterization of infectious hematopoietic necrosis virus of salmonid fish (Oregon strain). *Archiv fur die gesamate virusforschung*. 44:270-279.

McGarrity, G.C. (1982). Detection of mycoplasma infection of cell cultures. In *Advances in Cell Culture*, Vol. 2 (Maramorosch, K., ed.) pp.99-132. Academic Press, New York.

McGarrity, G.C., Sarama, J. & Vanaman, V. (1985). Cell culture techniques. *Nature*. 51(4):170-183.

McKenzie, R.A. & Hall, W.T.K. (1976). Dermal ulceration of mullet (*Mugil cephalus*). *Australian Veterinary Journal*. 52:230-231.

Meguro, Y., Nakai, T., Muroga, K. & Sorimachi, M. (1991). A cell line derived from the fin of Japanese flounder, *Paralichthys olivaceus*. *Fish Pathology*. 26(2):69-76.

Meier, W. (1985). Virale hämorrhagische septikämie: empfanglichkeit und

epizootiologische rolle des hechts (*Esox lucius* L.). *J. Appl. Ichthyol.* 1(4):171-177.

Menasveta, P. (1988). Current fish disease epidemic in Thailand. In *Practical Measures for Preventing and Controllong for Fish Diseases* (Sumawidjaja, K., Suwignyo, P., Tjitrosomo, S.S. & Umaly, R.C., eds.) pp. 115-127. Biotrop Spec. Publ. No. 28, Indonesia.

Menasveta, P., Dowrai, A., Piyatiratitivokul, S. & Singhasani, P. (1983). Acute toxicity of paraquat to some species of freshwater fish. *Proceedings of the Synposium on Freshwater Fish Epidemic: 1982-1983.* Chulalongkorn University, Bangkok.

Millar, S.D. (1994). Joint viral/fungal induction of EUS in snakeheads. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp. 272-278. Aquatic Animal Health Research Institute, Bangkok.

Miyazaki, T. (1994). Comparison among mycotic granulomatosis, saprolegniasis and anaaki-byo in fishes: a Japanese experience. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp 253-269. Aquatic Animal Health Research Institute, Bangkok.

Miyazaki, T. & Egusa, S. (1972). Studies on mycotic granulomatosis in freshwater fishes-I: the goldfish. *Fish Pathology.* 7:15-25.

Miyazaki, T. & Egusa, S. (1973). Studies on mycotic granulomatosis in freshwater fishes-IV: mycotic granulomatosis in wild fishes. *Fish Pathology.* 8:44-47.

Mohan, C.V. & Shankar, K.M. (1995). Role of fungus in epizootic ulcerative syndrome of fresh- and brackishwater fishes of India: a histopathological assessment. In *Diseases in Asian Aquaculture II* (Shariff, M., Arthur, J.R. & Subasinghe, R.P., eds.) pp. 299-305. Fish Health Section, Asian Fisheries Society, Manila, Philppines.

Mulcahy, D., Pascho, R.J. & Jenes, C.K. (1984). Comparison of *in vitro* growth characteristics of ten isolates of infectious hematopoietic necrosis virus. *J. gen. Virol.* 65:2199-2207.

Nelson-Rees, W.A. (1983). Karyological considerations and the history of cell culture. In *Uses and Standardization of Vertebrate Cell Culture* (Patterson, M.K., Jr., ed.) pp.142-151. Tissue Culture Association, Gaithersburg, Maryland.

Nelson-Rees, W.A., Daniels, D.W. & Flandermeyer, R.R. (1981). Cross-contamination of cells in culture. *Science.* 212:446-452.

Nicholson, B.L. (1989). Fish cell culture: an update. *Adv. Cell Culture.* 7:1-18.

Nicholson, B.L. & Byrne, C. (1973). An established cell line from the Atlantic salmon (*Salmo salar*). *J. Fish. Res. Board Can.* 30:913-916.

Nims, L., Fryer, J.L. & Pilcher, K.S. (1970). Studies on replication of four selected viruses in two cell lines derived from salmonid fish. *Proc. Soc. Exp. Biol. Med.* 135:6-12.

Nishizawa, T., Yoshimizu, M., Winton, J.R. & Kimura, T. (1991a). Comparison of genome size and synthesis of structural proteins of hirame rhabdovirus, infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus. *Gyobyo Kenkyu.* 26(2):77-81.

Nishizawa, T., Yoshimizu, M., Winton, J.R., Ahne, W. & Kimura, T. (1991b). Characterization of structural proteins of hirame rhabdovirus, HRV. *Dis. aquat. Org.* 10:167-172.

Noga, E.T. & Hartmann, J.X. (1981). Establishment of walking catfish (*Clarias batrachus*) cell lines and development of channel catfish (*Ictalurus punctatus*) virus vaccine. *Can. J. Fish. Aquat. Sci.* 38:925-930.

Nukwan, S., Lawanyawut, K., Tangtongpiros, M. & Veerasidth, P. (1990). Cross breeding between *Clarias macrocephalus* and *Clarias gariepinus*. *Proceedings of the 28th Kasetsart University Conference: Fisheries Section 29-31 January 1990.* Kasetsart University, Bangkok. (English abstract)

Nurtjahya, E. (1995). Plaque Assay of EUS-associated Rhabdoviruses. M.Sc. Dissertation, Institute of Aquaculture, University of Stirling, UK. 75 p.

ODA (1994). *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.). Aquatic Animal Health Research Institute, Bangkok.

Pearce, M. (1989). *Epizootic Ulcerative Syndrome Technical Report, Dec 1987-Sep 1989. Fishery Report No. 22*, Northern Territory Department of Primary Industry and Fisheries, Australia, 82 p.

Pichyangkura, S. & Bodhalamik, V. (1983). The study of *Achlya* sp. of fish disease in *Ophicephalus striatus*. *The Symposium on Fresh Water Fish Epidemic: 1982-1983.* Chulalongkorn University, Bangkok, pp. 197-205. (English Abstract)

Plumb, J.A., Bowser, P.R., Grizzle, J.M. & Mitchell, A.J. (1979). Fish viruses: a double-stranded RNA icosahedral virus from a North American cyprinid. *J. Fish. Res. Board. Can.* 36:1390-1394.

Rattanaphani, R., Wattanavijarn, W., Tesprateep, T., Wattanodorn, S., Sukolopong, V., Vetchagarun, S. & Eongpakornkeaw, A. (1983). Preliminary report on fish virus in snakehead fish. *Thai J. Vet. Med.* 13:44-50.

Reungprach, H., Boonyaratpalin, S., Supamataya, K., Kasornchandra, J., Polshewin, W. & Saduakdee, J. (1983). *Special Report of the Fish Disease Outbreak Committee in Thailand.* Ministry of Agriculture and Cooperatives, Bangkok, p. 64. (in Thai)

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cellular Biology.* 17:208-212.

Ristow, S.S., de Avila, J., LaPatra, S.E. & Lauda, K. (1993). Detection and characterization of rainbow trout antibody against infectious hematopoietic necrosis virus. *Dis. aquat. Org.* 15:109-114.

Roberts, R.J. (1993). Motile aeromonas septicaemia. In *Bacterial Diseases of Fish* (Inglis, V., Roberts, R.J. & Bromage, N.R., eds.) pp. 143-155. Blackwell Scientific Publication, London.

Roberts, R.J. (1994). Pathogenicity studies on fungi isolated from EUS. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp. 244-246. Aquatic Animal Health Research Institute, Bangkok.

Roberts, R.J., Frerichs, G.N., Tonguthai, K. & Chinabut, S. (1994). Epizootic ulcerative syndrome of farmed and wild fishes. In *Recent Advances in Aquaculture, Vol. 5* (Muir, J.F. & Roberts, R.J., eds.) pp. 207-239. Blackwell Science Ltd., Oxford, UK.

Roberts, R.J., MacIntosh, D.J., Tonguthai, K., Boonyaratpalin, S., Nuansri, T., Phillips, M.J. & Millar, S.D. (1986). *Field and Laboratory Investigations into Ulcerative Fish Diseases in the Asia-Pacific Region.* Technical Report RAS4508, FAO, Bangkok.

Roberts, R.J., Willoughby, L.G. & Chinabut,.S. (1993). Mycotic aspects of epizootic ulcerative syndrome (EUS) of Asian fishes. *J. Fish Dis.* 16:169-183.

Roberts, R.J., Wootten, R., MacRae, I.H., Millar, S.D. & Struthers, W. (1989). *Ulcerative Disease Survey, Bangladesh. Final Report to the Government of Bangladesh and the Overseas Development Administration.* Institute of Aquaculture, University of Stirling, UK. 104 pp.

Rovozzo, G.C. & Burke, C.N. (1973). *A Manual of Basic Virological Techniques.* Prentice-Hall Inc., Englewood Cliffs, New York.

Rukyani, A. (1994). Status of epizootic ulcerative disease in Indonesia. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp. 68-75. Aquatic Animal Health Research Institute, Bangkok

Saitanu, K., Wongsawang, S., Sunyascotcharee, B. & Sahaphong, S. (1986). Snakehead fish virus isolation and pathogenicity studies. In *The First Asian Fisheries Forum* (Maclean, J.L., Dizon, L.B. & Hosillos, L.V., eds.) pp. 327-330. Asian Fisheries Society, Manila, Philippines.

Schaefer, W.I. (1990). Terminology associated with cell, tissue and organ culture, molecular biology and molecular genetics. *In Vitro Cell. Dev. Biol.* 26:97-101.

Schwedler, T.E. & Plumb, J.A. (1982). *In vitro* growth kinetics and thermostability of the golden shiner virus. *J. Wildl. Dis.* 18:441-446.

Shea, T.B. & Berry, E.S. (1983). A serum-free medium that supports the growth of piscine cell culture. *In Vitro.* 11:818-824.

Shresta, G.B. (1994). Status of epizootic ulcerative syndrome (EUS) and its effects on aquaculture in Nepal. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp. 49-57. Aquatic Animal Health Research Institute, Bangkok.

Singhaseni, P. & Tesaprateep, T. (1985). Study on the histopathology of *Puntius gonionotus* exposed to paraquat. *Proceedings the Technical Conference on Living Aquatic Resources.* Chulalongkorn University, Bangkok.

Smith, S.A. (1992). Affinity purification of serum immunoglobulin from fish. In *Techniques in Fish Immunology* (Stolen, J.S., Flectcher, T.C., Anderson, D.P., Kaattari, S.L. & Rowley, A.F., eds.) pp. 125-130. SOS Publications, N.J.

Snieszko, S.F. (1974). The effects of environmental stress on outbreaks of infectious diseases of fishes. *J. Fish Biol.* 6:197-208.

Solis, J. & Moro, E.C. (1970). Viral susceptibility range of the fathead minnow (*Pimephalus promelas*) poikilothermic cell line. *Appl. Microbiol.*. 19(1):1-4.

Srisuparp, K., Laohawanit, O., Juingsamanyart, N., Limpoka, M., Karnchanomai, R., Pibul, S., Chaianan, C. & Phromsuthirak, P. (1984). *Studies on the mechanisms of the entry into fish body of Aeromonas hydrophila.* Special Report, Veterinary School, Kasetsart University, Bangkok, 14 p.

Subasinghe, R.P. (1993). Effects of controlled infections of *Trichodina* sp. on transmission of epizootic ulcerative syndrome (EUS) to naive snakehead, *Ophicephalus*

striatus Bloch. *J. Fish Dis.* 16:161-164.

Subasinghe, R.P., Jayasinghe, L.P., Balasuriya, K.S.W. & Kulathilake, M. (1990). Preliminary investigations into the bacterial and fungal pathogens associated with the ulcerative fish disease syndrome in Sri Lanka. In *The Second Asian Fisheries Forum* (Hirano, R. & Hanyu, I., eds.) pp. 655-657. Asian Fisheries Society, Manila, Philippines.

Subramanian, S., Chew-lim, M., Chong, S.Y., Howe, J., Ngoh, G.H. & Chan, Y.C. (1993). Molecular and electron microscopic studies of infectious pancreatic necrosis virus from snakehead. *IXth International Congress of Virology.* Glasgow, UK (Abstract), p. 368.

Supamataya, K., Boonyaratpalin, S. & Saduakdee, J. (1983). Relationship between water quality and sand goby (*Oxyeleotris marmoratus*) cultured in cage. *Special Report of the Fish Disease Outbreak Committee in Thailand.* Ministry of Agriculture and Cooperative, Bangkok, p. 164. (in Thai)

Supriyadi, H. (1988). Problems of bacterial fish disease and its control in Indonesia. In *Practical Measures for Preventing and Controlling Fish Diseases* (Sumawidjaja, K., Suwignyo, P., Tjitrosomo, S.S. & Umaly, R.C., eds.) pp. 111-113. Biotrop Spec. Publ. No. 28. Indonesia.

Tangtrongpiros, J., Thirapatsakun, T. & Wongsattayanont, B. (1983a). The examination and treatment of ectoparasites in *Trichogaster pectoralis* affected from an epidemic during December 1982 to February 1983. *The Symposium on Freshwater Fishes Epidemic: 1982-1983.* Chulalongkorn University, Bangkok, pp. 428-435. (English abstract)

Tangtrongpiros, J., Wattanavijarn, W., Thirapatsakun, T. & Wattanadorn, S.. (1983b). Fish kidney cell culture and cytopathic effects in the culture after infected with cell-free filtrate prepared from sick snakehead (*Ophicephalus striatus*). *Thai. J. Vet. Med.* 13(2):131-138. (English abstract)

Taylor, W.G. & Parshad, R. (1977). Peptones as serum substitutes for mammalian cells in culture. *Meth. Cell. Biol.* 15:421-434.

Thorgaard, G.H. & Disney, J.E. (1990). Chromosome preparation and analysis. In *Methods for Fish Biology* (Schrek, C.B. & Moyle, P.B., eds.) pp. 171-190. American Fisheries Society, Bethesda, Maryland.

Tonguthai, K. (1985). *A Preliminary Account of Ulcerative Fish Diseases in the Indo-Pacific Region.* National Inland Fisheries Institute, Bangkok.

Tonguthai, K., Chinabut, S., Limsuwan, C., Somsiri, T., Chanratchakool, P., Kanchanakhan, S. & MacRae, I.H. (1993). *Handbook of Hybrid Catfish: Husbandry and Health*. Aquatic Animal Health Research Institute, Bangkok.

Torres, J.L., Shariff, M. & Law, A.T. (1990). Identification and virulence screening of *Aeromonas* spp. isolated from healthy and epizootic ulcerative syndrome (EUS)-infected fish. In *The Second Asian Fisheries Forum* (Hirano, R. & Hanyu, I., eds.) pp. 663-666. Asian Fisheries Society, Manila, Philippines.

Towbin, H., Staehelin, T. & Gorgon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natl. Acad. Sci. U.S.A.* 76:4350-4354.

Tuan, P.A. (1994). Epizootic ulcerative syndrome in fish in Vietnam. In *ODA Regional Seminar on Epizootic Ulcerative Syndrome* (Roberts, R.J., Campbell, B. & MacRae, I.H., eds.) pp 76-81. Aquatic Animal Health Research Institute, Bangkok.

Tully, J.G. & Rasin, S. (eds.) (1983). *Methods in Mycoplasmology, Vol. II Diagnostic Mycoplasmology*. Academic Press, New York.

Tung, L.C., Chen, S.N. & Kou, G.H. (1991). Three cell lines derived from spleen and kidney of black porgy (*Acanthopagus schlegeli*). *Fish Pathology*. 26(3):109-118.

Wagner, R.R. (1987). Rhabdovirus biology and infection: an overview. In *The Rhabdoviruses* (Wagner, R.R., ed.) pp. 9-74. Plenum Press, New York.

Wagner, R,R, (1990). Rhabdoviridae and their replication. In *Virology*, 2nd edn. (Fields, B.N., Knipe, D.M., Chanock, R.M., Hirsch, M.S., Melnick, J.L., Monath, T.P. & Roizman, B., eds.) pp. 867-881. Raven Press, New York.

Wagner, R.R., Prevec, L., Brown, F., Summers, D.F., Sokol, F. & Macleod, R. (1972). Classification of rhabdovirus proteins: a proposal. *J. Virol.* 10:1228-1230.

Wagner, R.R., Synder, R.M. & Yamazaki, S. (1970). Proteins of vesicular stomatitis virus: kinetics and cellular sites of synthesis. *J. Virol.* 5:548-558.

Watson, M.L. (1958). Staining of tissue sections for electron microscopy with heavy metals. *Journal of Biophysics, Biochemistry and Cytology*. 4:475-478.

Wattanavijarn, W., Ousavaplangchai, L., Rattanaphani, R., Sukolapong, V., Tesaprateep, T., Eongpakornkeaw, A., Tangtrongpiros, J., Vetchangarun, S. & Thirapatsakun, T. (1983a). Virus-like particles in the skeletal muscle, capillaries and spleen of sick snakehead fish (*Ophicephalus striatus*) during a disease epidemic. *Thai. J. Vet. Med.* 13(2): 122-130.

Wattanavijarn, W., Tesaprateep, T., Wattanodorn, S., Sukolapong, V., Vetchangarun, S. & Eongpakornkeaw, A. (1983b). Virus-like particles in the liver snakehead fish. *Thai J. Vet. Med.* 13(1): 51-57.

Wattanavijarn, W., Rattanaphani, R., Tesaprateep, T., Tangtrongpiros, J., Thirapatsakun, T., Sukolapong, V., Eongpakornkeaw, A. & Vetchangarun, S. (1984a). Examination of sick snakehead fish during a disease epidemic by scanning and transmission electron microscopy. *Thai. J. Vet. Med.* 14(1): 13-21.

Wattanavijarn, W., Tangtrongpiros, J., Rattanaphani, R., Sukolapong, V., Eongpakornkeaw, A. & Vetchangarun, S. (1984b). Examination of sick catfish by scanning and transmission electron microscopy. *Thai J. Vet. Med.* 14(1): 31-38.

Wattanavijarn, W., Tangtrongpiros, J. & Wattanodorn, S.(1986). Viruses of ulcerative diseased fish in Thailand, Burma and LAO P.D.R. In *First International Conference on the Impact of Viral Diseases on the Development of Asian Countries.* WHO. (Abstract) p. 121.

Wattanavijarn, W., Tangtrongpiros, J., Wattanodorn, S. & Hunnak, P. (1985). Detection of viruses from cell cultures by using electron microscope and induction of disease by infected cell culture fluid. *Thai. J. Vet. Med.* 36:143-151.

Wattanavijarn, W., Torchy, C., Tangtrongpiros, J. & de Kinkelin, P. (1988). Isolation of a birnavirus belonging to Sp serotype, from southeast Asia fishes. *Bull. Eur. Ass. Fish Pathol.* 8(5):106-108.

Wiktor, T.J., György, E., Schlumberger, H.D., Sokol, F. & Koprowski, H. (1973). Antigenic properties of rabies virus components. *Journal of Immunology.* 110:269-276.

Willoughby, G.R., Roberts, R.J. & Chinabut, S. (1995). *Aphanomyces invaderis* sp. nov., the fungal pathogen of freshwater tropical fish affected by epizootic ulcerative syndrome. *J. Fish Dis.* 18:273-275.

Winton, J.R. (1992). Evolution of fish rhabdoviruses. In *Salmonid Diseases: Proceeding of the OJI International Symposium on Salmonid Diseases* (Kimura, T., ed.) pp. 88-95. Hokkaido University Press, Sapporo, Japan.

Winton, J.R., Lannan, C.N., Fryer, J.L., Hedrick, R.P., Meyers, T.R., Plumb, J.A. & Yamamoto, T. (1987). Morphological and biochemical properties of four members of a novel group of reoviruses isolated from aquatic animals. *J. gen. Virol.* 68:353-364.

Wolf, K. (1973). Freshwater fishes. In *Tissue Culture Methods and Applications* (Kruse, P.F. Jr. & Patterson, M.K. Jr., eds.) pp. 138-143. Academic Press, New York.

Wolf, K. (1984). Fish viruses: their biology, classification, hosts, pathology and control. In *Control of Virus Diseases* (Kurstak, E. & Marusyk, R.G., eds.). Marcel Dekker Inc., New York.

Wolf, K. (1988). *Fish Viruses and Fish Viral Diseases.* Cornell University Press, Ithaca, New York.

Wolf, K. & Ahne, W. (1982). Fish cell culture. In *Advances in Cell Culture,* Vol. 2 (Maramorosch, K., ed.) pp. 305-327. Academic Press, New York.

Wolf, K. & Mann, J.A. (1980). Poikilotherm vertebrate cell line and viruses: a current listing for fish. *In Vitro.* 16:168-179.

Wolf, K. & Quimby, M.C. (1962). Established eurythermic line of fish cells *in vitro. Science.* 135:1065-1066.

Wolf, K. & Quimby, M.C. (1969). Fish cell line and tissue culture. In *Fish Physiology,* Vol. 3 (Hoar, W.S. & Randall, D.J., eds.) pp. 253-305. Academic Press, New York.

Wolf, K. & Quimby, M.C. (1973). Fish viruses: buffers and methods for plaquing eight agents under normal atmosphere. *Appl. Microbiol.* 25:659-664.

Wolf, K. & Quimby, M.C. (1976a). Primary monolayer culture of fish cells initiated from minced tissues. In *TCA Manual 2* (Evans, V.J., Perry, V.P. & Vincent, M.M., eds.) pp. 445-448. Tissue Culture Association, Rockville, Maryland.

Wolf, K. & Quimby, M.C. (1976b). Primary monolayer culture of fish cells initiated from trypsinized tissues. In *TCA Manual 2* (Evans, V.J., Perry, V.P. & Vincent, M.M., eds.) pp. 453-456. Tissue Culture Association, Rockville, Maryland.

Wolf, K. & Quimby, M.C. (1976c). Procedures for subculturing fish cells and propagating fish cell lines. In *TCA Manual 2* (Evans, V.J., Perry, V.P. & Vincent, M.M., eds.) pp. 471-474. Tissue Culture Association, Rockville, Maryland.

Wolf, K., Quimby, M.C., Pyle, E.A. & Dexter, R.P. (1960). Preparation of monolayer cell culture from tissues of some lower vertebrates. *Science.* 132:1890-1891.

Wunner, W.H. & Peters, D. (1991). Family *Rhabdoviridae.* In *Classification and Nomenclature of Viruses* (Francki, R.I.B., Fauquet, C.M., Knudson, D.L. & Brown, F., eds.). *Arch. Virol. Suppl..* 2:250-262.

Yamamoto, T., Batts, W.N. & Winton, J.R. (1992). *In vitro* infection of salmonid

epidermal tissues by infectious hematopoietic necrosis virus and viral hemorrhagic septicemia virus. *J. Aquat. Animal Health.* 4:231-239.

Appendix 1 Estimation of 50% end-points by the Spearman-Kärber method (Kärber, 1931).

A reliable method which is suitable for use with relatively small numbers of test subjects provided that:

a) The dilution factor is constant, so that the interval between successive logarithmic doses is constant.

b) The dilution range covers the whole of the response rate from 0% to 100%, or is so close to it that the next dilution in the series can safely be assumed to be 0% or 100%.

$$\text{Mean log TCID}_{50}\ (m) = x + 1/2(d) - d \sum (r/n)$$

Where

x = log of the highest reciprocal dilution.

d = log of the dilution interval.

r = no. of test subjects not infected at any dilution.

n = no. of test subjects inoculated at any dilution.

The method also enables an estimate of the standard error of the end-point to be made from which 95% confidence limits can be derived.

Variance of mean (Vm)

When the same number of test subjects are used at each dilution level.

$$Vm = d^2 / [n^2(n\text{-}1)] \sum [r(r\text{-}n)]$$

Variance of mean (Vm)

When a different number of test subjects are used at each dilution level.

$$Vm = d^2 \sum \{[r(r\text{-}n)] / [n^2(n\text{-}1)]\}$$

Standard error of mean (SE_m)

$$SE_m = \sqrt{V_m}$$

95% confidence limits

95% confidence limits means that it is 95% certain that the true end-point result lies between these limits. If the experiment was to be repeated in the same way on 100 occasions, it could be confidently expected that on 95 occasions the result obtained would fall within these limits.

$$95\% \text{ confidence limits} = m \pm 2(SE_m)$$

The above calculation procedure is a copy from part of the lecture note of Fish Virology taught in Aquatic Pathobiology Course at the Institute of Aquaculture by Dr. G.N. Frerichs.

Example: Rhabdovirus strain T9412 titration in BF-2 cells (Chapter 4).

Culture no.	Virus dilution				
	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
1	+	+	+	-	-
2	+	-	-	-	-
3	+	+	+	+	-
4	+	+	+	-	-
5	+	+	-	-	-
6	+	+	-	-	-

+ = infected culture − = uninfected culture

Reciprocal dilution	10^6	10^7	10^8	10^9	10^{10}
Log_{10} reciprocal dilution	6	7	8	9	10
No. of uninfected cultures (r)	0	1	3	5	6
No. of cultures inoculated (n)	6	6	6	6	6

Mean $\log_{10}$ $TCID_{50}$ (m) $= x + 1/2\,(d) - d\sum(r/n)$

$= 10 + 1/2(1) - 1(1/6 + 3/6 + 5/6 + 6/6)$

$= 8$

Mean titre (m) $= 10^8$ $TCID_{50}$ / volume inoculated

The volume inoculated was 0.1 ml, then the titre could be expressed as 10^8 $TCID_{50}$/0.1 ml, or 10^9 $TCID_{50}$/ml, or 9 $\log_{10}$ $TCID_{50}$/ml. The virus titre presented in this study was

shown only as mean virus titre (m). However, a 95% confidence limit could be calculated as follows.

Variance of mean (Vm) $= \{d^2 / [n^2(n-1)]\} \sum [r(r-n)]$

$= \{1^2 / [6^2(6-1)]\} [1(6-1) + 3(6-3) + 5(6-5) + 6(6-6)]$

$= (1/180)\ (19)$

$= 0.1056$

Standard error of mean (SE_m) $= \sqrt{V_m}$

$= \sqrt{0.1056}$

$= 0.325$

95% confidence limits $= m \pm 2(SE_m)$

$= 9 \pm 2\ (0.325)$

$= 9 \pm 0.650$

Virus titre $= 9 \pm 0.65\ \log_{10}\ TCID_{50}/ml$

Appendix 2 Estimation of 50% lethal dose (LD_{50}) of rhabdovirus T9412 against snakehead fish fry.

LD_{50} was calculated similar to the $TCID_{50}$ in Appendix 1. The stock rhabdovirus was propagated in HCK line and had a titre of 7.83 $\log_{10}$ $TCID_{50}$/ml.

$$\text{Mean log } LD_{50}\ (m) = x + 1/2(d) - d\,\Sigma\,(r/n)$$

Where

x = log of the highest reciprocal dilution.

d = log of the dilution interval.

r = no. of test subjects not infected at any dilution.

n = no. of test subjects inoculated at any dilution.

Virus dilution	1/20000	1/40000	1/80000	1/160000	1/320000	1/640000	1/1280000
log reciprocal dilution	4.3	4.6	4.9	5.2	5.5	5.8	6.1
no. of fish not infected	0	0	0	0	0	19	20
no. of fish used	20	20	20	20	20	20	20

LD_{50} value was found between log reciprocal dilution 5.5 and 5.8.

$$\text{Mean log } LD_{50}\ (m) = 6.1 + 1/2(0.3) - 0.3\ (19/20 + 20/20)$$

$$= 6.1 + 0.15 - 0.585 = 5.67$$

$$LD_{50} = 10^{5.67}$$

The value indicated that the dilution of stock rhabdovirus T9412 at $10^{-5.67}$ or 1/467735 was able to kill 50% of snakehead fish fry under the test conditions (section 4.4.5, Chapter 4).

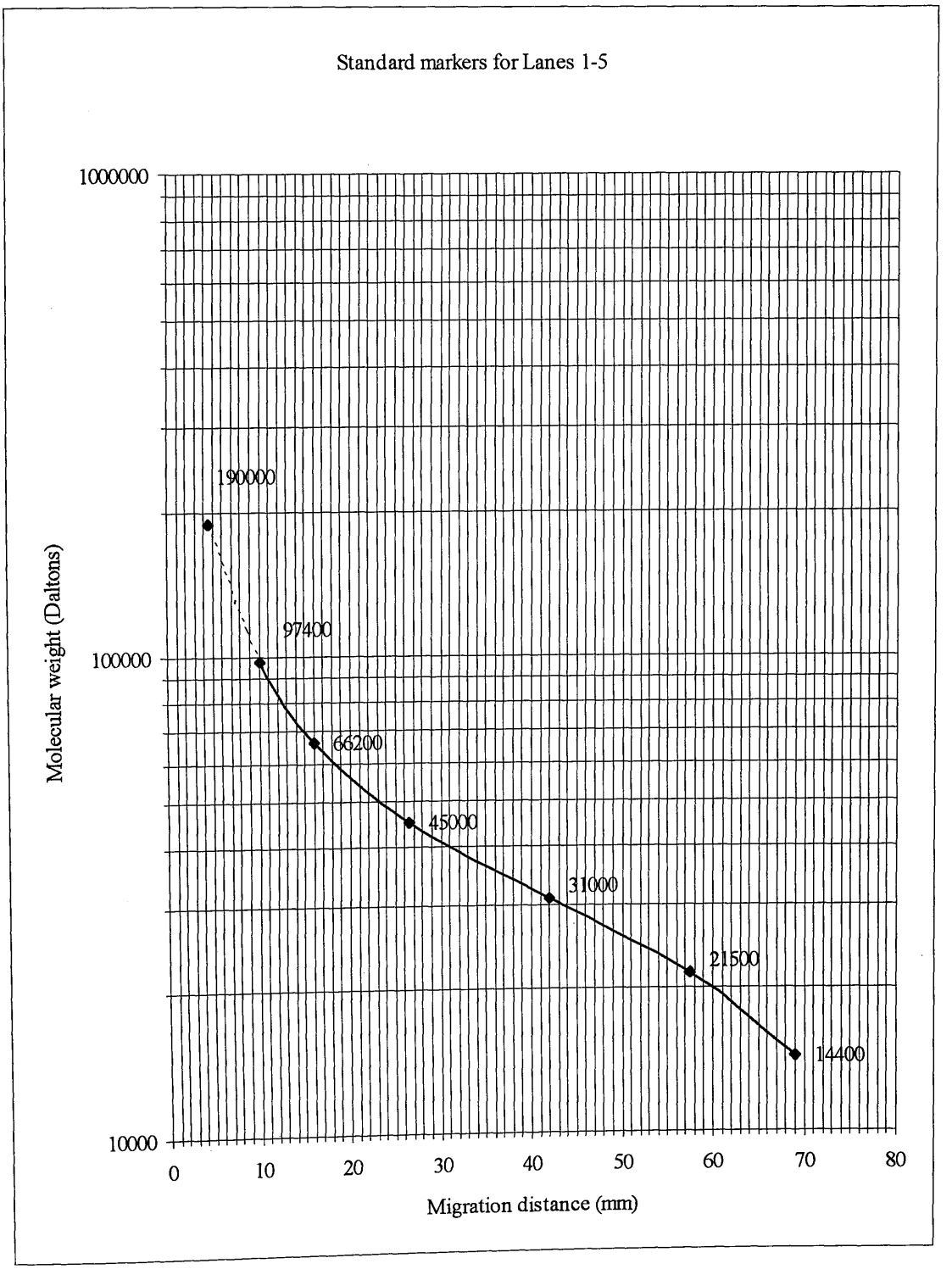
stock virus dilution at $10^{-7.83}$ = 1 $TCID_{50}$

stock virus dilution at $10^{-5.67}$ = $\dfrac{1 \times 10^{-5.67}}{10^{-7.83}}$

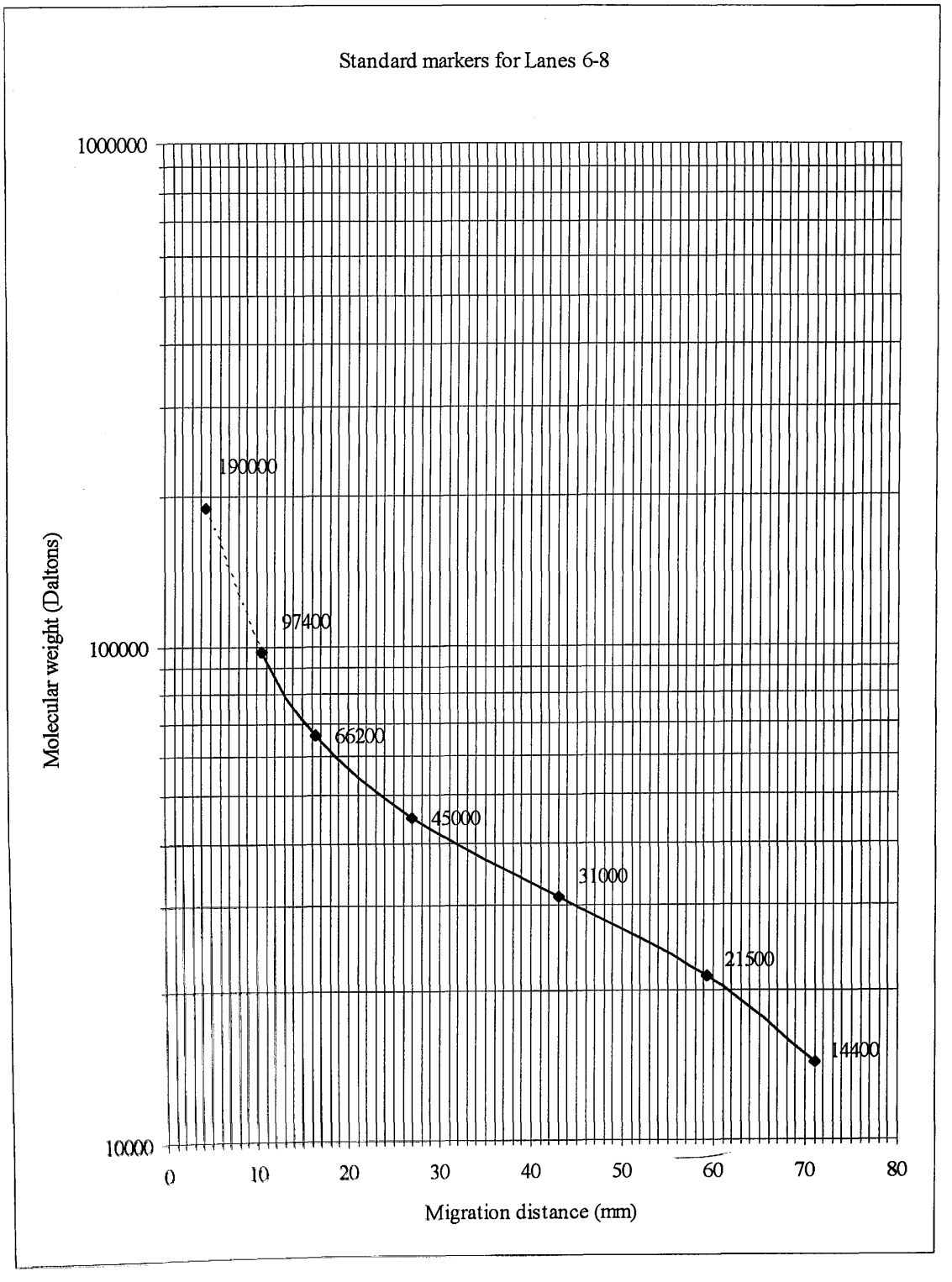
= $10^{2.16}$ $TCID_{50}$

LD_{50} = $10^{2.16}$ $TCID_{50}$/ml or 2.16 $\log_{10}$ $TCID_{50}$/ml

Appendix 3 Standard mobility curves of molecular weight markers in 12% SDS-PAGE. Distances of protein migration in the gel were measured between the middle of the band and the top edge of resolving gel and plotted on a semilogarithmic scale. Left marker lane was assigned as the reference for lanes 1-5 and right marker lane assigned as reference for lanes 6-8.



Appendix 3 (cont.)



Note: Molecular weight markers (BioRad, USA) contain 97.4 kDa phosphorylase B, 66.2 kDa serum albumin, 45.0 ovalbumin, 31.0 kDa carbonic anhydrase, 21.5 kDa trypsin inhibitor and 14.4 kDa lysozyme.
Protein at 190.0 kDa is a possible location of structural protein L of rhabdovirus on the standard curves.