

**OVERSEAS DEVELOPMENT ADMINISTRATION
NRED AQUACULTURE RESEARCH PROGRAMME**

Studies on virus infections of food fish in the Indo-Pacific region

ODA RESEARCH PROJECT R5430

FINAL REPORT

by

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Executive summary

To investigate the significance of viruses isolated from fish affected by epizootic ulcerative syndrome (EUS), a series of viral characterization and pathogenicity studies were undertaken. Further investigation of outbreaks of EUS and non-EUS diseased fish were undertaken with regard to viral involvement.

A rhabdovirus (T9412) was demonstrated as a primary pathogen for striped snakehead fish and a combination of this virus and *Aphanomyces* spp. fungi appeared to produce more severe EUS disease than a single infection with the fungus. Improved virus isolation methodology resulted in higher viral recovery rates from EUS outbreaks.

The genome of the snakehead cell line associated retrovirus was fully sequenced and cloned and demonstrated to be distinct from other gene sequenced retroviruses.

The EUS associated reovirus (T9231) was shown to be a member of the genus *Aquareovirus*, family *Reoviridae*, but was a new serotype. Experimental studies with this virus in snakehead fish demonstrated non-pathogenicity. The blotched snakehead (BSN) cell line associated virus was shown to be a new member of the family *Birnaviridae* and was also shown to be non-pathogenic for snakehead fish. Enzyme-linked immunosorbent assays (ELISA) were developed for both the aquareovirus and the BSN virus and new cell lines developed from Asiatic snakehead fish.

A regime to remove mycoplasma contamination from cell lines was developed and demonstrated as effective.

The piscine neuropathy nodavirus was isolated from diseased spotted brown grouper from Thailand.

Background

It has been widely recognised that disease is the most significant limiting factor in the development of food fish culture, as has been dramatically illustrated by the annually recurring outbreaks of the epizootic ulcerative syndrome (EUS) which has now become established throughout the Indo-Pacific region. The rational implementation of methods of treatment and control of infectious fish disease requires a thorough understanding of the aetiology, epidemiology and pathogenesis of such conditions and the availability of rapid and accurate means of diagnosis of the clinical and latent states of infection.

At the ODA Regional Seminar on Epizootic Ucerative Syndrome, held in Bangkok in January 1994, it was recognised that although the clinical condition was characterised by the presence of an invasive *Aphanomyces* spp. infection, there was a distinct and critical pre-mycotic stage which demanded further investigation. In addition, as studies had shown the presence of a diverse range of viral agents in fish affected with EUS, it was recommended that further work be undertaken to determine the nature and distribution of tropical food fish viruses throughout the region.

Studies previous to this research period had indicated that at least three groups of viruses had been identified in some fish suffering EUS. These were classified as belonging to the families *Birnaviridae*, *Rhabdoviridae* and *Reoviridae*. The significance of these viruses in the induction of clinical disease remained to be established.

Project purpose

The two main objectives of the project were as follows:

- (i) to continue virological studies on outbreaks of EUS to examine the hypothesis that a specific viral infection is the underlying primary cause of the disease condition and
- (ii) to identify and characterize viral agents isolated from food fish in the Indo-Pacific region, to establish baseline data on the incidence of viral infections in tropical fish species.

More specifically the plan of work for the project was to conduct research in the following areas:

- ?? further virological analysis of material from EUS outbreaks
- ?? virological analysis of material from non-EUS disease problems, when opportunities arose
- ?? characterization of viral agents associated with EUS and non-EUS disease
- ?? investigations of the relationships between the tropical fish viruses and those from temperate fish species
- ?? pathogenicity studies with selected viral isolates

It was envisaged that through these areas of research a greater understanding and increased knowledge, of the role the viruses play in the EUS and other disease conditions, would be achieved. From this knowledge should flow means to rapidly diagnose, prevent and control such conditions in the future.

Research activities

The following were the main areas of research activity for the reporting period:

- ?? fish tissue samples from two 1994 outbreaks of EUS in Thailand were virologically analysed at both the Aquatic Animal Health Research Institute (AAHRI), Bangkok and the Institute of Aquaculture (IOA), Stirling. An anomalous result between the two centres was initially obtained and this led to further investigation into improving the methodology for virus isolation in outbreaks of EUS.
- ?? re-examination of fish tissue extracts sampled throughout 1985 to 1994 was undertaken. This included some 100 samples from Bangladesh, India, Nepal and Thailand which were inoculated onto cell lines not previously used to determine a) whether the rhabdovirus strains isolated earlier were still viable after long term low temperature storage in tissue extracts and b) whether other viruses might have been present which were not isolated on the cell lines first used.
- ?? the previously observed cell culture derived snakehead retrovirus (SnRV) was studied through experimental infection studies, in snakehead fish at AAHRI and in *Puntius* sp. at IOA, to determine whether the SnRV could be integrated into the genome of live EUS-susceptible fish. The complete nucleotide sequence for the SnRV genome was established in collaboration with the University of Glasgow Veterinary School and the relationship of this agent to other retroviruses analysed by examining the alignments of the reverse transcriptase amino acid sequences from a representative range of retroviruses for which such data is available.
- ?? characterization studies of the rhabdovirus isolates from EUS outbreaks in Thailand in 1994 were undertaken. Similar studies of an EUS-associated reovirus isolate and a

reovirus-like agent from a persistently infected blotched snakehead (BSN) cell line were conducted and these compared to four aquareoviruses from the USA and Europe.

?? experimental infections were conducted for the EUS-associated reovirus isolate with snakehead fish and a number of detection techniques developed or improved.

?? mycoplasma infections of a variety of fish cell lines were investigated through various detection methods and a number of treatment regimes conducted and compared for efficacy.

?? following isolation of the piscine neuropathy nodavirus in 1995, characterization studies were undertaken and further isolations attempted from fish tissues obtained from various countries including Thailand.

All the areas of proposed research activity were investigated and the planned inputs, including investigations of suspect viral disease in non-EUS diseased fish were also achieved.

Outputs

a) The rhabdovirus isolates from the EUS outbreak in Thailand in 1994 were found to be antigenically similar to the 1992 isolates although there were differences in virus growth rates and cell culture susceptibilities between the 1994 strains themselves. In brief the rhabdovirus strain T9412 caused death in fry and skin damage in juvenile striped snakehead fish. A combination of this rhabdovirus and pathogenic *Aphanomyces* spp. fungus appeared to induce more severe EUS disease in snakehead fish than a single infection with the fungus. Three characterised rhabdovirus strains (T9415, T9416 & 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 ulcerative disease rhabdovirus (UDRV) strains SL 11, BP and 20E possessed vesiculovirus-like electrophoretotypes. The lyssavirus-like EUS-associated rhabdoviruses, except strain T9416, were structurally and serologically similar and the “serotype Sh” is proposed for this group, 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. This research was conducted as part of the PhD programme for Somkiat Kanchanakhan and published in 1996.

Although a causal link between a rhabdovirus infection and the clinical manifestation of EUS had been effectively discounted some years ago on the basis of a very low and inconsistent isolation rate of the virus and failure to experimentally reproduce the condition by direct exposure to the putative pathogen, the reappearance of the agent in samples taken in the 1994 outbreaks and additional factors which emerged

from the concurrent studies in AAHRI and IOA indicated that the virus merited further consideration.

The additional factors that emerged were:

- (i) higher virus isolation rates were obtained when tissues were processed and inoculated into cell cultures within 24 hours of sampling without storage or transport
- (ii) virus could only be isolated from fish during the early stages of a disease outbreak and not from the same population two weeks later
- (iii) virus could be isolated directly from skin lesion material, demonstrating local association of the virus with the lesion
- (iv) virus could be isolated from more than one fish species (snakehead and gourami) in the same environment, at the same time.

b) re-examination of 100 selected fish tissue extracts from SE Asian freshwater species and mullets from southern India, affected with ulcerative disease conditions, did not result in any further virus isolations. There was no apparent survival of EUS-associated rhabdoviruses in original tissue extracts after prolonged storage at low temperatures.

c) the cloning and complete sequencing of the snakehead retrovirus (SnRV) genome demonstrated that it was only distantly related to mammalian C-type retroviruses and of no relation to the oncogenic and immunodeficiency viruses pathogenic to higher vertebrates. SnRV also appears divergent from walleye dermal sarcoma virus (WDSV), the only other fish retrovirus for which sequence data is available. The findings from this research were published in the *Journal of Virology* (Hart *et al.* 1996).

In the study to determine whether cell culture derived SnRV could be integrated into the genome of live fish, molecular examination of tissues obtained at intervals over 10 weeks from SnRV inoculated *Puntius* sp. showed no evidence for virus integration into living fish. Virus integration could also not be demonstrated in striped snakehead fish inoculated at AAHRI. These findings lend further support to the contention that the cell culture derived retroviruses are non-pathogenic for snakehead fish and are not implicated in the pathogenesis of EUS.

d) the EUS associated reovirus (T9231) was confirmed by nucleic acid analysis as belonging to the genus *Aquareovirus*, family *Reoviridae* and was determined to be a new serotype, unrelated to other members of this genus. Experimental infection studies revealed that the virus persisted in snakehead tissues for at least eight weeks and that circulating antibodies were produced by the fish in response to infection.

e) what was originally considered to be a reovirus-like agent in a persistently infected blotched snakehead (BSN) cell line, was shown to be a new member of the family *Birnaviridae*. It was demonstrated as differing from two other members of this family, the infectious pancreatic necrosis virus (IPNV) and the sand goby virus (SGV), by SDS-PAGE and genome segment analysis. Experimental transmission of the BSN virus to snakehead fish was achieved through i/p injection and minor histopathological changes were seen in the spleen, kidney and liver cells.

f) sandwich ELISAs were developed for the reovirus and the BSN virus to detect both agents in tissues. That developed for the BSN virus gave good results, however for the reovirus cross reactions masked the virus presence. A new cell culture initiated from the Asiatic snakehead fish was found to support replication of the BSN virus. The work

summarised in parts d), e) and f) will form part of the PhD thesis for Mr. J. K. Riji-John who will submit his dissertation in September 1997.

g) a regime to remove mycoplasma contamination from valuable cell cultures, used in isolation and pathogenicity studies, proved to be successful in all but one case. This makes the deposition in culture collections and the availability of these cell culture systems to a wider range of scientific workers more feasible. The results of of this study were published in the Journal of Fish Diseases (Frerichs 1996).

h) the piscine neuropathy nodavirus (PNN), the causal agent of viral nervous necrosis (VNN) or viral encephalopathy and retinopathy, was isolated for the first time in the world using a snakehead cell culture system, developed through the ODA funded research. The identity of the nodavirus was confirmed physically and biochemically. The virus was also isolated from stored tissue material taken from diseased spotted brown grouper in Thailand. This was obtained with the co-operation and assistance of AAHRI and NICA in Thailand. Further isolated material from Japan and Singapore confirmed the potential of this system for diagnostic, pathogenicity and epidemiological studies. Characterization of the nodavirus isolates has revealed serological differences between isolates and *in vitro* storage analysis has highlighted the stability of the virus outside the fish host. This initial work has been published in the Journal of General Virology (Frerichs *et al.* 1996). The snakehead cell line has also been deposited in the European Collection of Animal Cell Cultures (ECACC) and from there is available to laboratories throughout the world.

Contribution of outputs

The investigations into EUS associated viruses revealed a range of potential candidates which could initiate the skin and muscle invasion by the *Aphanomyces* spp. fungi leading to clinical lesions. It has been shown that although the aquareovirus and the BSN virus are probably new serotypes of their respective groups, in experimental transmission studies these viruses have not been demonstrated to be primary pathogens. Their involvement in a more complex multifactorial syndrome, such as EUS, cannot be discounted at this time. The fact that these are distinctly new viral types in both cases is an important contribution to baseline knowledge of viruses associated with food fishes in the region.

The rhabdovirus strain T9412 was demonstrated as a potential primary pathogen for juvenile striped snakehead fish and a combination of this virus and *Aphanomyces* spp. fungi appear to induce more severe EUS disease in snakehead fish than a single infection with the fungus. The results from this study are significant and indicate that the rhabdovirus could be one of a complex of aetiological agents for EUS and at least two serotypes of EUS-associated rhabdoviruses have been identified. In the light of these important findings, this is an area that requires further investigation through fish infectivity trials and pathogenesis studies.

Some of the recent information obtained through this research was disseminated during a regional workshop entitled "Cell culture and virology techniques" held at AAHRI in March 1996 and run jointly by AAHRI and IOA staff.

The isolation of the piscine neuropathy nodavirus has been a very significant breakthrough in fish disease research and opens up a vista of opportunities for the

diagnosis, control and prevention of this devastating disease of marine fish, both farmed and wild. The specific cell line utilised for the isolation of the virus is available to other fish disease centres through the ECACC. The areas of priority for nodavirus research are:

i) characterization and identification of the virus from different species and countries which should include antigenicity and immunogenicity, sensitivity to heating, drying, chemical disinfection and diagnostic methods

ii) epidemiology - where are the viral reservoirs? What are the modes of transmission?

What makes fish susceptible?

iii) immunology - what is the response of fish to viral infection and what are the prospects of vaccination?

Publications during the reporting period

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