REPORT ON PROJECT:

(89/32) Identification of eggs of commercially important fish species and further studies for diagnosis of fish pathogens using monoclonal antibody technology.
Project Leaders: Assoc. Prof. P. J. Hanna and Dr. L. S. Hammond

Previous Reporting of Results for Project 89/32

Some of the results were presented to a FIRDC meeting held in Melbourne on 22 November, 1990. Additional results were included as a background to a 1991 application to the FIRDC.

Objectives

The objectives were to produce monoclonal antibodies (mAbs) for management of a range of problems in Australian fisheries and aquaculture, and to specifically apply these techniques to:

- detection, monitoring and control programs for *Aeromonas* infections causing furunculosis in wild and cultured fish,
- rapid identification of tuna eggs and other commercially important species, and
- maintain hybridomas that identify pathogenic *Vibrio* species, produced under a previous FIRDC grant, and complete the transfer of technical knowledge to relevant user groups.

Background

At the start of the project we had already developed, with FIRDC funding, the world's best panel of mAbs for the rapid identification of *Vibrio* pathogens. The hybridomas producing these mAbs were to be maintained frozen in liquid nitrogen throughout the new project and negotiations made with the new FIRDC for their transfer to commercial use.

An integral part of the new research project was to identify fish eggs using mAbs. It was considered by fisheries scientists and managers to offer significant opportunity for understanding the biology of commercially important species. Unambiguous identification of eggs would enable data to be gathered, or inferences to be drawn, on reproductive

output, spawning location, survival of eggs, dispersal of eggs, etc. There were no methods available for the identification of eggs of most fish species. Only a few relatively common and well-known species were identifiable in which the eggs had exceptional characteristics (eg. special pigmentation, size or disposition of internal features). The aim was to develop methods to identify the eggs of some commercially important fish species, particularly the yellowfin tuna (*Thunnus albacares*) and barramundi (*Lates calcarifer*). The direction was followed, partly in response to requests from the previous FIRDC.

Another main component of the new research project was to produce mAbs for the rapid identification of *Aeromonas salmonicida,* a pathogenic bacteria causing furunculosis disease in fish, particularly salmonids. The bacteria was present in Australia as atypical *A. salmonicida* and was originally introduced in cultured goldfish as goldfish ulcer disease (GUD). The virulence of these introduced strains are currently unknown. We planned to produce mAbs specific to either the lipopolysaccharide of *Aeromonas salmonicida*, or more important, the A-protein surface array which is regarded as a factor contributing to virulence. The mAbs were to then be used in rapid diagnostic situations.

Summary of Results

(a) Identification of *Aeromonas salmonicida*, a pathogen of wild and cultured fish.

Development of monoclonal antibodies against virulent strains of *Aeromonas salmonicida*, commonly isolated from infections of salmonid and other fishfish, was successful. The results are documented in two manuscripts (see attachments 1-2). The first concerns problems we overcame during standard identification of strains and the second refers to one mAb that specifically reacts with the A-protein of *A. salmonicida.* They are:

Altmann, K., Marshall, M., Nicholson, S. E., Hanna, P. J. and Gudkovs, N. (1992). Glucose repression of pigment production in atypical isolates of *Aeromonas salmonicida* responsible for gold fish ulcer disease (GUD). Microbios, 72, 215-220.

Chai, Z., Cartwright, G., Altmann, K., Marshall, M. and Hanna, P. J. (1993). A-protein of *Aeromonas salmonicida* atypical strains: immunoidentification and studies of its release. In revision.

In brief, *A. salmonicida* isolates and extracted A-protein solution were supplied by the Australian Fish Health Reference Laboratory, now relocated from Benalla to AAHL (CSIRO, GEELONG). BALB/c mice were immunised with an formalin-killed atypical *A. salmonicida* isolate 85:9370-A (an A-protein producing strain) together with 40μg of A-protein, extracted and purified according to the methods of Phipps *et al.* (1983). Spleens from hyperimmune mice were then fused with SP2/O myeloma cells using polyethylene glycol, following the methods of De St. Groth and Scheidegger (1980).

The fusion resulted in the isolation of 22 hybridomas showing three patterns of specificity. Seventeen hybridomas produced monoclonal antibodies reacting with the extracted A-protein only. Three hybridomas produced monoclonal antibodies reacting with the extracted A-protein, as well as with one or more *Vibrio* or *Aeromonas* isolates; unfortunately none of these were specific to *A. salmonicida.* Two hybridomas produced monoclonal antibodies which reacted with a broad spectrum of bacteria, including the original isolate and with the A-protein extract. However, none of the mAbs recognised only isolate 85:9370-A and other *A. salmonicida* strains having an A-protein surface array. This problem was thought to be due to denaturation of the A-protein during the extraction process, or formalin fixation on the bacterial surface, thereby changing the immunological identity. Consequently, the mAbs that were produced did not react with native A-protein on live cells.

Another fusion was performed in which the atypical *A. salmonicida* isolate 85:9370-A (an A-protein producing strain) that was initially killed with sodium azide prior to immunising mice. Positive hybridomas with mAbs reacting with 85:9370-A, and other *A. salmonicida* isolates, were rescreened against a wide range of additional gram-negative bacteria, including different *Aeromonas* species. One mAb (F19P512E11D), isotyped as IgG_1/κ, reacted with only *A. salmonicida* isolates having A-protein. It was then characterised in a number of tests and proved to be useful in the identification *A. salmonicida* isolates.

(b) ***Identification of tuna eggs and other commercially important species.***

Surveys had shown that most commercial fish eggs collected from ocean trawls were routinely stored in ethanol for subsequent laboratory studies. We had already shown in a preliminary test on frozen cultured Barramundi eggs that antisera could be developed against the eggs.

Therefore, the first research investigated the development of a antisera against ethanol-fixed Barramundi egg membranes. This was achieved by using egg membranes which were thawed, then thoroughly washed three times in PBS to remove egg yolk, and fixed in 70% ethanol for one week. The membranes were broken by sonnication, centrifuged and then homogenised in 0.9% saline. Protein content of the fish membrane preparation was then determined before immunising BALB/c mice three times on a weekly basis with 60μg protein/injection of ethanol-fixed membranes. Other mice were immunised in a similar manner with 41μg protein/injection of unfixed membrane preparations. The end-point titres of antisera were high enough to allow the antisera to be used in FITC immunofluorescence studies of egg-membranes. Antisera against the ethanol-fixed eggs showed a strong FITC reaction while the antisera against the frozen eggs was weaker. The study showed that it was possible to obtain an immune response from washed and ethanol-fixed membranes of fish eggs.

Although tuna fish eggs were the first choice for making antibodies against them, the first samples collected by Dr G. McPherson were lost in a CSIRO freezer malfunction and it since then it has been impossible to obtain further samples. This unavailability has been due to a number of factors including lack of knowledge about timing of egg maturity, the place where tuna spawn, and to research vessel schedules and costs. Samples of tuna eggs were sought from various places but with no success. After discussions with Dr M. Walker (Executive Officer of FIRDC) and Dr R. Thresher (CSIRO, Division of Sea Fisheries) it was planned that skip jack mackerel eggs were to be supplied by the Division of Sea Fisheries, but again, none were forthcoming due to the inability to collect them. The same occurred in our attempts to obtain cultured snapper eggs from Cronula. The overall lesson was that it was almost impossible to obtain pure samples of most commercial Australian marine fish. Increased aquaculture activities may change this in the future.

Therefore we continued with development of monoclonal antibodies against eggs of *Lates calcarifer*, the barramundi, a species of commercial significance. Screening of all antibodies with eggs was carried out using two indirect immunofluorescence assays, firstly with anti-mouse FITC and then with anti-mouse rhodamine. As well, indirect ELISA, electrophoresis (ie. SDS-PAGE) and western blotting were performed. Fish eggs used in the tests were from Barramundi, Blue-eye Trevalla (obtained from local fish suppliers), Mauhi (obtained from Hawaii) and Murray Cod (obtained from the Snob's Creek

Hatchery). Some autofluorescence was observed but the intensity was low and the colour was pale yellow, unlike that of the two fluorescing fluorochromes used in the study.

The screening results were disappointing due to obvious 'false-positives' obtained when testing whole eggs. For example, it was found that positive results occurred when the mAbs were tested with whole eggs, but so did the secondary anti-mouse conjugates in both indirect immunofluorescence assays and ELISA. In fact the results obtained with use the secondary conjugates in controls was produced stronger results than when the mAbs were incorporated in the indirect assays. This indicated that the whole fish eggs possessed substances that reacted with the anti-mouse antibodies conjugated to the FITC or rhodamine fluorochromes, or enzymes. Normally these anti-mouse antibodies would have only attached to the eggs coated with monoclonal antibodies as a secondary reaction.

Therefore, analyses of the eggs were carried out using electrophoretic separations of egg substances, combined with immunoblotting, to determine which substance(s) were producing the 'false-positives'. Electrophoresis using SDS-PAGE of the egg membranes and yolk produced protein profiles in which there was considerable protein variation between the species (Fig 1a). The distinctive molecule of approximately 66 kDa molecule occurring in the yolk is presumed to be albumin. It also occurs in the egg-membrane profiles and is therefore considered to be hard to remove during preparation of membranes. The distinct variation between the egg types could possibly be expanded to analysis the eggs from additional closely related species and eggs collected from trawls. Immunoblotting of the proteins showed that there were several components that reacted with the antibodies (Fig 1b), and the 66 kDa molecule presumed to be albumin, would account for much of the 'false-positives'.

In summary, the application of mAb technology to the identification of fish eggs has been fraught with two major problems, namely:

- the difficulty to obtain fish eggs from field collections or aquaculture, and
- false positives obtained through the reaction of anti-mouse antibodies with the eggs.

The research using mAb technology to the identify prawn larvae and fish pathogens has certainly been more fruitful.

Figure 1. Analyses of proteins of membranes and yolk of fish eggs. Lanes: T, Blue-eye Trevalla; M, Mauhi; C, Murray Cod and B, Barramundi. Molecular weight (kDa) markers are indicated on the left hand side.

Fig. 1a. Electrophoresis using SDS-PAGE of the egg membranes and yolk, and Coomassie blue staining.

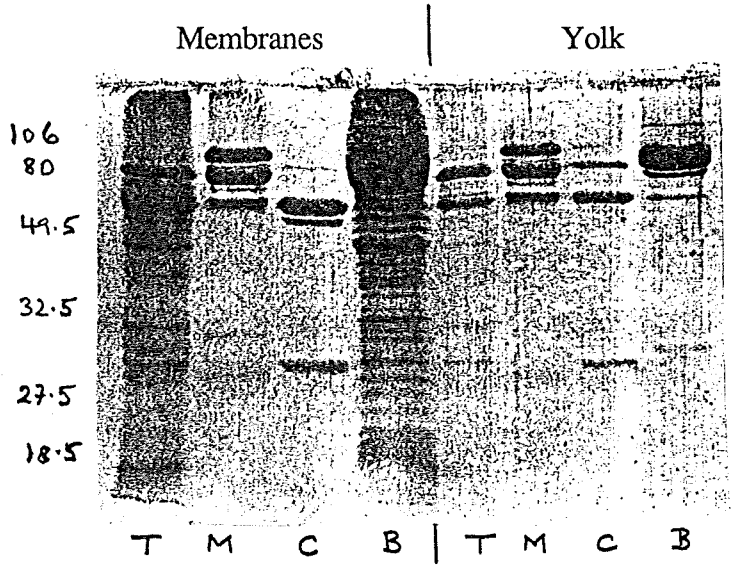
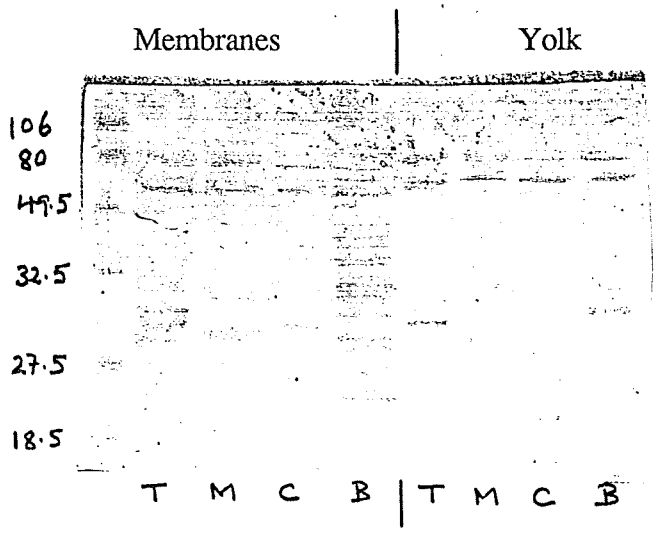


Fig. 1b. Immunoblotting of proteins from egg membranes and yolk with enyme conjugate only.



(c) Maintenance of hybridomas produced to identify pathogenic *Vibrio* species and the transfer of technical knowledge to relevant user groups.

The hybridomas produced to identify pathogenic *Vibrio* species were successfully maintained. In addition, negotiations were initiated for a joint agreement between Deakin

University and the FIRDC to licence relevant companies to use the hybridomas for the production and marketing of diagnostic mAbs. Results of the research involving the production of mAbs to identify *Vibrio* and marine larvae were compiled into manuscripts and published (see attachments 3-7). These included:

Hanna, P. J., Altmann, K., Chen, D., Smith, A., Cosic, S., Moon, P. and Hammond L. S. (1992). Development of monoclonal antibodies for the rapid identification of epizootic *Vibrio* species. J. Fish Dis. 15, 63-69.

Chen, D., Hanna, P. J., Altmann, K., Smith, A., Moon, P. and Hammond, L. S. (1992). Monoclonal antibodies identifying *Vibrio* species commonly isolated from infections of humans, fish and shellfish. Environ. Appl. Microbiol. 58, 3694-3700.

Chen, D. and Hanna, P. J. (1992). Attachment of *Vibrio* pathogens to cells of *Oncorhynchus mykiss* (Richardson 1836), the rainbow trout. J. Fish Dis. 15, 331-337.

Hanna, P. J., Richardson, B. J., Altmann, K., Roper, K. G., Smith, J. M. and Hammond, L. S. (1993). The Production of Monoclonal Antibodies for Use as Probes in the Identification of Northern Australian Crown-of-Thorns Starfish and Commercial Prawn Larvae. In: Dispersal and Recruitment in the Marine Environment. Sammarco, P. W. and Heron, M. (eds.). American Geophysical Union, Space Science Institute, San Antonio.

Criteria for Evaluation

The project has been evaluated, and:

- monoclonal antibodies have been produced to identify virulent A-protein positive strains of *Aeromonas salmonicida*, but not the target fish eggs, due to wide cross-reactivities with eggs of other species,
- sufficient screening has been undertaken to confirm the specificity of the *Aeromonas salmonicida* antibodies,
- relevant user groups are aware of the existence of the techniques, and some have had the opportunity to collaborate in their use,
- the results have been submitted to the scientific literature, and
- steps have been taken or planned to enable further development of the results in industry situations.

Transfer of Results to Industry

(a) Notification of Availability of the Technique

Notification of results of the project has occurred through the publication of papers to scientific journals (see attachments).

Results of the production of diagnostic monoclonals against *Aeromonas salmonicida*, the causative agent of goldfish ulcer disease (GUD) and furunculosis or ulcerative disease of salmon, were presented together with other work on pathogenic *Vibrio* species, to the:

- Annual Conference of the Australian Mariculture Association, Brisbane, July 1989,
- University as a Departmental seminar, November 1989,
- Annual Conference of the Australian Society of Microbiology, Launceston, July 1990, and
- 1st Symposium on Diseases in Asian Aquaculture, Bali, November 1990.

Additional presentation of the results, together with results of recent research, are planned for the forthcoming 1993 meetings of the Australian Marine Sciences Association, the 2nd Symposium on Diseases in Asian Aquaculture and the Australian Microbiology Society.

(b) Direct Access to Methodology

Some people involved in fisheries biology, management and disease control will be provided with antibodies (eg. AFHRL). Assistance with immunodiagnostic testing has been given by the research staff. However, it is expected that most access will occur through joint marketing of the diagnostics.

(c) Marketable Diagnostic Kits or Research Antibodies

An agreement for the joint ownership of the *Aeromonas* and *Vibrio* diagnostics have been drawn up on a 50:50 basis between the Fisheries Research and Development Corporation and Deakin University. The transfer of technology in this form requires a joint venture with other parties (eg. CSL Ltd, Biotechnology Australia Ltd or other major distributor) to develop and market products. This has been slow to eventuate, due primarily to the corporatisation of CSL. However, talks are now progressing with major diagnostic-producing and distributing companies and the FRDC will be kept up-to-date with the progress.