Immunodiagnosis of virulent strains of *Aeromonas hydrophila* associated with epizootic ulcerative syndrome (EUS) using a monoclonal antibody.

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Running title: Immunodiagnosis of *Aeromonas hydrophila*

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; HAT, hypoxanthine-aminopterin-thymidine; LPS, lipopolysaccharide; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, tris buffered saline.

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Abstract. A virulent strain of *Aeromonas hydrophila* associated with epizootic ulcerative syndrome (EUS) was used to produce monoclonal antibodies that identified only virulent strains of *A. hydrophila*. Antibodies from a clone, designated as F26P5C8, were found to identify the *A. hydrophila* isolates taken from EUS fish, and which were found to be virulent using inoculation tests. Immunodiagnosis of a large number of *A. hydrophila* from Australia and Japan, showed some additional isolates to be identified by F26P5C8 but the status of their virulence presently unknown.

Introduction

Aeromonas hydrophila, a member of the family *Vibrionaceae*, is a gram negative motile rod, having the capacity to infect cold-blooded vertebrates and mammals, and exist freely in water (Ho, Mietzner, Smith & Schoolnik, 1990). It is a primary and secondary pathogen of a number of aquatic and terrestrial animals including humans (Howard & Buckley, 1985). It is considered to be the principal cause of bacterial haemorrhagic septicaemia in fresh water fish (Frerichs, 1989), and has been reported in association with various ulcerative conditions\syndromes including 'epizootic ulcerative syndrome' (EUS) in Thailand and the Philippines (Lio-Po, Albright & Alapide-Tendencia, 1992; Llobrera & Gacutan, 1987) and 'red spot' disease in Australia (Cahill, 1987). Numerous species have been infected, including snakehead (*Ophicephalus striatus*), catfish (*Clarias batrachus*), gouramy (*Trichogaster pectoralis*), crucian carp (*Carassius carassius*), goby (*Glossogobius giurus*), sea mullet (*Mugil cephalus*), flat-tailed mullet (*Liza dussumieri*), bream (*Actanthopagrus australis*), threadfin (*Polydactylus sheridani*), barramundi (*Lates calcifer*), rainbow trout (*Oncorhynchus mykiss*), trout cod (*Maccullochella macquariensis*) and Maquarie perch (*Macquaria australasica*) (Lio-Po *et al.*, 1992; Llobrera & Gacutan, 1987; Cahill, 1987). In most disease incidences, and particularly the Australian ones, the virulence of each isolate was not determined and the role of *A. hydrophila* as a primary or secondary pathogens has not been established.

Inoculation studies with isolates from EUS fish have shown significant variation in the relative pathogenicity of isolates (Torres, Shariff & Tajima, 1992). A number of avirulence determinants have been identified and examined (reviewed by Cahill, 1990). They include: the production of endotoxins such as lipopolysaccharides (LPSs), extracellular enterotoxins, hemolysins, cytotoxins, extracellular proteases, aerolysin, pili, adherence to cells and the possession of an S-layer surface protein. However, there remains a clear need for the development of tests for markers of virulence, in order to differentiate between pathogenic, and less, or non-pathogenic isolates.

The importance to differentiate between pathogenic and non-pathogenic isolates of *A. hydrophila* has lead to the development of serological subgrouping schemes. For example, virulent strains have been shown to constitute a single serogroup that is based on properties of the O antigen (Mittal, Lalonde, Leblanc, Oliver & Lallier, 1980). Subsequent studies indicated that virulent *A. hydrophila* strains possessed a common O antigen (Lallier, Mittal, Leblanc, Lalonde & Oliver, 1981) associated with homogeneous length polysaccharide chains (Dooley, Lallier, Shaw & Trust, 1985). Agglutination assays have indicated that virulent *A. hydrophila* isolates, associated with EUS, belong to serotype I (Torres *et al.*, 1992). However, additional serogrouping of Aeromonads have been unable to associate virulence of isolates with serogroups (Sakazaki & Shimada, 1984; Misra, Shimada, Bhadra, Pal & Nair 1989).

This paper reports on a monoclonal antibody raised against *A. hydrophila* isolated from EUS fish and discusses its potential use in identifying potentially pathogenic *A. hydrophila* isolates.

Materials and methods

Bacterial strains

A. hydrophila isolates used in this study to produce MAbs and carry out initial screenings (Table 1) were supplied from overseas in 2% (v/v) formalin sources to

comply with quarantine regulations. Some additional isolates were supplied as live cultures from the Australian Fish Health Reference Laboratory (AFHRL), Geelong, including 85:584-1A and 85:8438. A wide range of gram-negative bacteria were used in screening MAb specificities and included strains of *A. caviae, A. salmonicida,* and *A. sobria*. A further 68 isolates of *A. hydrophila* (Table 2) were screened at the AFHRL and followed by another 43 *A. hydrophila* isolates at Hokkaido University.

The bacteria were grown aerobically, with agitation, at 37°C in Tryptone Soya Broth (Oxoid) to log phase, firstly in 10mL volumes and subsequently in 10mL volumes. They were then stored at 4°C in 2% (v/v) formalin until required. Prior to use, the cell suspensions were washed three times in PBS by centrifugation at 13,500 rpm in an MSE microfuge, to remove the formalin.

Generation of hybridomas

Due to its virulence and association with EUS, *A hydrophila* 45 (Torres *et al.*, 1990), was selected for immunization and the subsequent production of hybridomas. The immunization protocol of Carlin and Lindberg (1983) was used. Briefly, approximately $2x10^8$ cells in 500µL PBS was resuspended (1:1) in Freunds Complete Adjuvant (CSL) and 500µL of the resulting emulsion injected intraperitoneally (*i.p.*) into 6-8 week old female Balb/c mice. On day 9, the treatment was repeated using Freunds Incomplete Adjuvant (CSL). Four days prior to the production of hybridomas, mice were given a booster *i.p.* injection of 10^8 bacteria in PBS.

Media and culture conditions

All cell lines were cultured in RPMI 1640 medium (Flow Laboratories) supplemented with 10% (v/v) foetal calf serum (CSL) 1mM glutamine (Flow Laboratories) and antibiotics (50 I.U. penicillin and 50cmg streptomycin per mL; Cytosystems) in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Production of hybridomas

Immune splenocytes from mice immunized with *A. hydrophila* 45 were fused with the non-immunoglobulin secreting murine HGPRT-deficient myeloma cell line Sp2/0-Ag.14 (Schulman, Wilde & Kohler 1978) as described by de St. Groth & Scheidegger (1980) using a fusogen of 45% (w/v) PEG 4000 (Merck) in distilled water containing 5% (v/v) dimethyl sulphoxide (Merck). The PEG solution was prepared on the day of fusion, so as to limit the formation of toxic peroxides, and sterilized by passage through a 0.45µm filter (Millipore). Hybridomas were selected by growth in HAT medium (Littlefield 1964) using murine peritoneal macrophages as feeder cells. To ensure monoclonality, hybridomas of interest were cloned by limiting dilution (Goding 1980).

Isotyping

Monoclonal antibodies were isotyped using an Amersham isotyping kit (Amersham) according to the manufacturer's instructions.

Screening and selection of hybridomas

Following HAT selection, hybridoma supernatants were screened against the immunizing bacterial isolate by indirect immunofluorescence as described below. The specificity of hybridomas selected was then determined by both indirect immunofluorescence and ELISA using a large panel of bacterial isolates (Table 1).

Indirect immunofluorescent antibody staining

Five microlitres of bacterial strains, containing 10^8 cells/mL in PBS, were added to wells of multiwell slides (Flow Laboratories). After air drying, cells were heat-fixed and 10µL of test or isotype control hybridoma supernatants were added to the appropriate wells of the slides. Following incubation at RT for 30 min in a humidified chamber, slides were washed thoroughly with PBS and incubated at room temperature for 30 min with 20µL of sheep anti-mouse-rhodamine conjugate (Silenus, Code DF) diluted 1:50 in PBS containing 1% (w/v) BSA. The slides were then washed 3 times

in PBS and mounted using 90% (v/v) glycerol and 4% (v/v) propyl gallate in PBS. Slides were examined for specific immunofluoescence using a Zeiss epifluorescence microscope.

ELISA screening

The specificity of hybridomas was examined by ELISA, as described by Carlin and Lindberg (1983). Briefly, bacterial isolates were washed three times in 0.05M carbonate buffer pH 9.6, adjusted to a final optical density of 0.4Å (=620nm), and 100µL added to appropriate wells of a 96-well microtitre plate (Disposable Products, Code No. 24139). The bacteria were left to adsorb overnight at 4°C before the plates were washed 6 times with 0.9% (w/v) NaCl containing 0.05% (v/v) Tween 20 (Selbys), and then blocked by the addition of 100µL of PBS containing 1% (w/v) BSA (Sigma, A-7888) at 37°C for 2h. Plates were then washed 6 times as above, and 100µL of hybridoma supernatant was added to the appropriate wells. Following incubation at RT for 1h, plates were again washed 6 times and 100µL of goat anti-mouse alkaline phosphatase conjugate (Sigma A-0162), diluted 1:1000 in PBS containing 1% (w/v) BSA was added to each well, and incubated at 37°C for 1h. Following a further 6 washes, bound antibody was detected by the addition of 100µL of substrate solution containing 1mg/mL *p*-nitrophenyl phosphate (Sigma) prepared according to the manufacturer's instructions. Following incubation at 37°C for 100min, absobance was quantified at 405nm using a Flow Mutiscan MCC/340 Microtitre plate reader.

Characterization of the antigenic epitope

To determine the heat stability of the antigenic epitope recognized by F26P5C8 antibodies, preparations of sodium azide killed or formalin-fixed *A. hydrophila* in 0.5M sodium carbonate buffer, pH 9.6, were boiled in a water bath for 2h and then analyzed by indirect immunofluorescence and ELISA.

Lipopolysaccharide (LPS) was prepared from 5L TBS cultures of *A. hydrophila* isolates, 85:584-1A (ELISA positive) and 85:8438 (ELISA negative). The LPS was isolated using the hot phenol-water extraction method as previously described (Westphal, Luderitz & Bister, 1952) and purified by ultracentrifugation (Westphal, Jann, & Himmelspach, 1983). Contaminating proteins were removed from LPS-containing samples by centrifugation at 104,000g for 3h at 4°C in a Beckman TY-65 rotor using a Beckman model L5-65 centrifuge. The procedure was repeated until no contaminating protein was detected by absorbance readings at 280nm. The LPS was then freeze-dried, made up to 1mg/mL in sterile distilled H_2O, and stored at -70°C until required.

SDS-PAGE

SDS-PAGE was performed using a mini protean II electrophoresis system (Bio-Rad) according to the method of Laemmli (1970). Initially, *A. hydrophila* strains at 0.4Å (=620nm), were washed three times in PBS by repeated centrifugation at 13,000rpm for 5min in a Table MSE microcentrifuge. The final pellets were then resuspended 1:1 in SDS-PAGE reducing sample buffer, vortexed vigorously, boiled for 3min and centrifuged as above to remove any cell debris. Twenty microlitre samples were separated electrophoretically using 12.5% reducing gels at a constant current of 10mA per gel until the bromophenol blue dye front had reached the bottom of the gels. Resolved proteins were visualized with silver stain (Tsai and Frasch, 1982). Molecular weights were estimated using SDS-PAGE standards (Bio-Rad, low molecular weight range, 71330).

Immunoblotting

Following SDS-PAGE, resolved components in unstained gels were transferred onto nitrocellulose sheets (Bio-Rad) at 30V overnight (Towbin, Staehelin and Gordon, 1979). Unbound reactive sites on the nitrocellulose sheets were blocked with 1% (w/v) BSA in TBS containing 10mM Tris-HCl and 50mM NaCl, pH 7.4, for 1h. Sheets

were then incubated for 1h with either test or control hybridoma supernatant and washed three times in TBS containing 0.05% (v/v) Tween 20. Sheep anti-mouse horseradish peroxidase conjugate (Silenus, Code DAH) diluted 1:2000 in TBS containing 1% (w/v) BSA was then added and the sheets incubated at RT for 1h. Following a further six washes, bound antibodies were detected by development in a substrate solution of 0.03% (w/v) 4-chloro-1-naphthol (Sigma) in TBS containing 20% (v/v) methanol and 0.015% (v/v) H_2O_2 until dark purple bands appeared.

Results and discussion

While no EUS outbreaks have been reported in Australia, it is not known whether these isolates are all ready present in Australia as the potential for pathogenic *A. hydrophila* strains to enter Australia exists. It is therefore important that current quarantine practices are able to recognize these isolates. No reliable diagnostic tests are currently available to screen *A. hydrophila* isolates. The present study was therefore initiated to develop a monoclonal antibody reactive with pathogenic *A. hydrophila* isolates from South East Asia.

Production of monoclonal antibodies

The highly virulent *A. hydrophila* isolates 5 and 45, implicated with epizootic ulcerative disease (EUS) in South East Asia were imported with other *Aeromonas* isolates in formalin. Of the two most virulent isolates, *A. hydrophila* 45 from an EUS-positive Philippine wild *Clarias* sp. was selected for the production of monoclonal antibodies.

Forty hybridomas were generated, of which, one clone designated as F26P5C8, was found to bind strongly to *A. hydrophila* isolate 45. Antibodies secreted by this hybridoma were found to be of the IgG_3,λ isotype. F26P5C8 also recognzied three other *A. hydrophila* isolates, *viz.* EUS fish isolate 5, human diarrhea isolate Ah15 and isolate 85:584-1A. Other *A. hydrophila* and gram negative bacteria were negative in the cross-screening tests.

Screening of Malaysian and Philippine isolates from infected and healthy fish

In order to determine whether F26P5C8 was specific for pathogenic *A. hydrophila* and *A. hydrophila*-like isolates, a large panel of isolates, from both healthy and infected fish in the Philippines and Malaysia, were screened (Table 1). The virulence of these isolates had been previously determined by injecting healthy fish with $6.4x10^5$ cells of each isolate (Torres *et al.*, 1990). An important finding of the current study was that F26P5C8 not only detected the immunizing isolate, 45, but also isolates 5, 11, 42, 43 and 46, which were all shown to be virulent in inoculation studies. Three additional isolates, 25, 32, and 33, were detected by F26P5C8 and despite them coming from healthy fish were subsequently shown to be virulent in inoculation tests. Therefore the current immunlogical tests confirmed the data from the experimental infections.

The avirulent *A. hydrophila* isolates, 1 and 44, from an infected fish were also reactive to F26P5C8. This may suggest that the isolates were in fact virulent but further testing is required. In another test, F26P5C8 reacted with an *A. hydrophila*-like isolate, 57, which was virulent in inoculation tests. This isolate may truly be an *A. hydrophila* isolate, but additional testing is required to confirm its taxonomic status.

Screening Japanese A. hydrophila *isolates from fish and human sources*

A panel of 43 Japanese *A. hydrophila* isolates from various healthy and diseased fish from species such as carp (*Cyprinus carpio*), ayu (*Plecoglossus altivelis*), eel (*Anguilla* sp.) catfish, (*Clarias* sp.) and yamabe (*Oncorhynchus masou*), or the rearing water, was screened using F26P5C8. None of 16 isolates from diseased fish were recognized by F26P5-C8. Two isolates, Ah-90 isolated from yamabe intestine and Ah-88 from eel rearing water were recognized by F26P5C8. The virulence of these two isolates is unknown. It would therefore be an important next step to determine virulence and make comparisons with those from Malaysia and the Philippines.

It is interesting to note that F26P5C8 recognized four of nine human *A. hydrophila* isolates, Ah-13, Ah-14, Ah-15 and Ah- 16, from cases of diarrhea. One isolate of *A.*

caviae and 10 isolates of *A. sobria* from human diarrhea tested negative. These data suggest a possible etiological link between fish and humans, reinforcing the need for a quick and accurate diagnosis of *A. hydrophila*. Not all isolates of *A. hydrophila* from humans were detected, indicating that the antigen recognized by F26P5C8 was not present on some *A. hydrophila* isolates or that these isolates were not the primary cause of the diarrhea.

Screening isolates held at the Australian Fish Health Reference Laboratory, CSIRO

A panel of 70 *A. hydrophila* isolates from Australian freshwater and imported ornamentals were screened using F26P5C8 supernatant by indirect immunofluorescence. Of these, eight isolates were found to react strongly, while a further two produced a weak reaction with F26P5C8 (Table 2). Interestingly, only one (ie. 86:5879-G), of four isolates obtained from different Barramundi from the same region and on the same date, reacted with F26P5C8, indicating the heterogenous nature of *A. hydrophila* populations, even in a localized area. A very weak cross-reactivity was first observed for an *A. sobria* isolate by indirect immunofluorescence but was eliminated following western blot analysis of whole cell lysates. These studies of Australian isolates show that they were capable of being detected with F26P5C8 and have epitopes cross-reactive with virulent EUS isolates from Malaysia and the Philippines. Virulence status of the positive Australian isolates has not been determined.

Characterization of antigenic surface antigen

Western blots using whole cell lysates of *A. hydrophila* isolates held at the Australian Fish Health Reference Laboratory, CSIRO, revealed that F26P5C8 antibodies recognized all isolates positive by indirect immunofluorescence, including those isolates scored as weak positives (Fig. 1). Ten isolates negative by indirect immunofluorescence were examined as negative controls and showed no activity.

The activity of F26P5C8 in western blots was such that the supernatant at low dilution resulted in intensely stained blots with little detail. As a result considerable difficulty was encounted in obtaining blots which showed sufficient detail to make clear comparison between the isolates. Most of the antibody activity was observed in the high molecular weight range greater than 50kDa. Despite the diverse geographic and host range of fish from which the isolates were obtained there was a remarkable degree of similarity in the patterns observed. All the isolates displayed common bands of similar molecular weights and staining intensities. The binding pattern produced was considered characteristic for the group in general, suggesting that antigens expressed by these bacteria were shared and hence the bacteria though to be closely related.

Heat stability of the antigen recognized by F26P5C8 antibodies on the surface of EUS *A. hydrophila* isolates 5, 15, 45 and 85:584-1A was determined after boiling isolates for 2h. All were positive in indirect immunofluorescence tests, whereas isolates Ah86 and Ah138, which acted as controls, were negative. These data indicated that the antigenic determinant was heat stable and probably LPS rather than protein.

Conclusions

It has been suggested that the most virulent strains of *A. hydrophila*, which are also associated with EUS, belong to serotype I (Torres et al., 1992). While F26P5C8 antibodies appear to be specific for this serotype, they do not recognize all serogroup I isolates. Other serogroups were negative (Table 1). The detection of virulent strains indicates that the use of monoclonal antibodies may provide information with regard to pathogenicity of *A. hydrophila*.

While differences between the LPSs of virulent and non-virulent strains of *A. hydrophila,* have been identified, the S-layer protein on the surface has been considered to be a better indicator of potential virulence than LPS endotoxins (Cahill,

1990). Further research is required to ascertain the meaning of these properties in terms of pathogenicity.

Acknowlegments

We gratefully acknowledge Dr. J. L. Torres for permission to use his *A. hydrophila* isolates. This work was funded by the Fishing Industry Research and Development Council.

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Table 1. Comparison of virulence of *A. hydrophila* isolates from Malaysia and the Philippines with reactivity to F26P5C8 by indirect immunofluorescence and ELISA

Isolate	Serotype*	Fish condition*	Virulence*			Immunofluorescence	ELISA
			high	weak	avirulent		
A. hydrophila							
5	I	D	+			+	+
11	I	D	+			+	+
42	I	D	+			+	+
45	I	D	+			+	+
46	I	D	+			+	+
9	IV	D		+		-	-
13	III	D		+		-	-
43	I	D		+		+	+
1	II	D			+	+	+
44	II	D			+	+	+
24	II	H	+			-	-
29	II	H	+			-	-
32	I	H	+			+	+
33	I	H	+			+	+
25	U	H		+		+	+
26	II	H			+	-	-
27	II	H			+	-	-
30	II	H			+	-	-
34	II	H			+	-	-
71	U	H			+	-	-
A. hydrophila-like							
3	V	D		+		-	-
8	IV	D		+		-	-
15	V	D		+		-	-
57	VII	D		+		+	+
60	U	D		+		-	-
4	U	D			+	-	-
6	U	D			+	-	-
56	U	D			+	-	-
63	U	D			+	-	-
31	U	H			+	-	-
70	V	H			+	-	-
73	U	H			+	-	-
74	U	H			+	-	-
76	U	H			+	-	-

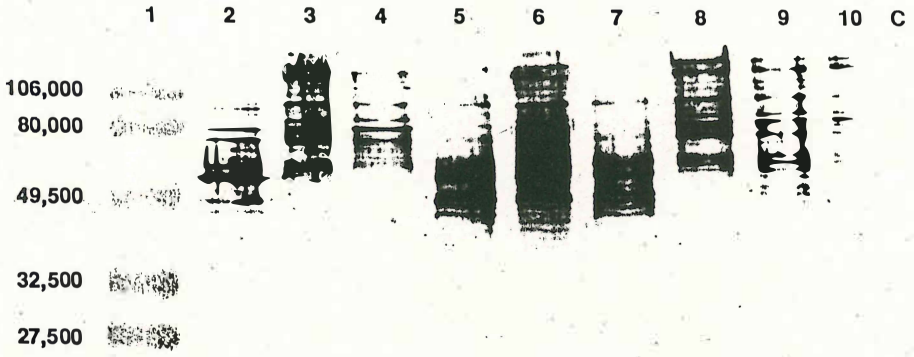
* Torres *et al.* (1990); U, Unassigned serotype or untested; D=diseased fish, H=healthy fish; Virulence was determined by injecting 6.4×10^5 cells into healthy fish

Table 2. Indirect immunofluorescence screening of live Australian *A. hydrophila* isolates from clinical samples by using F26P5C8 antibodies

Isolate[a]	Source	Indirect Immunofluorescence[b]
85:584-1A	*Carassius auratus* L. (Gold fish)	+
86:5879-G	*Lates calcarifer* Bloch (Barramundi)	+
87:7281-6A	*Oncorhynchus mykiss* Richardson (Rainbow trout)	+
88:737-B	*Salmo salar* L. (Brown trout)	+
172	*Helostoma temmincki* Cuvier and Valeniennes (Kissing Gourami)	+
84:12235-2F	*Salmo salar* L. (Atlantic salmon)	+
83:1164511-2A	*Salmo salar* L. (Atlantic salmon)	+
84:12235-8F	*Salmo salar* L. (Atlantic salmon)	+
258	*Salmo salar* L. (Atlantic salmon)	+(weak)
259	*Salmo salar* L. (Atlantic salmon)	+(weak)
85:8438 (control)	*Carassius auratus* L. (Goldfish)	-

a, Isolates held at the Australian Fish Health Reference Laboratory, CSIRO, Geelong
b, Tests of 59 additional *A. hydrophila* isolates were negative.

Fig. 1. Immunoblotting of lysates, prepared from *A. hydrophila* isolates held at the Australian Fish Health Reference Laboratory, with F26P5C8 antibodies. Prestained molecular weight standards (Bio-Rad) were run on lane 1. Lysate samples of isolates were loaded and run on lanes 2-10, in the same order as listed in Table 2. Thus lane 11 (C) acted as a negative control.


1
2
3
4
5
6
7
8
9
10
C
106,000
80,000
49,500
32,500
27,500

REPORT ON PROJECT:
(91/31) Monoclonal antibodies for the identification of fish pathogens and fish eggs
Project Leader: Assoc. Prof. P. J. Hanna

Objectives

These were to produce monoclonal antibodies for management of problems in Australian fisheries and aquaculture, and to specifically apply these techniques to:
(a) detection, monitoring and control programmes for virulent strains of *Aeromonas hydrophila* commonly associated with epizootic ulcerative syndrome (EUS) outbreaks, and
(b) rapid identification of eggs of commercially important fish species (e.g. snapper).

Background

We had already developed the world's best panel of monoclonal antibodies for the rapid identification of *Vibrio* pathogens, and atypical strains of *Aeromonas salmonicida*, the causative agent of goldfish ulcer disease (GUD). Others had been developed against barramundi eggs, but further cross-screening against other fish eggs was needed to establish their specificities. This information was presented to the FIRDC meeting held in Melbourne on 22 November, 1990.

Our research planning and strategies were shown to be well founded by the information presented in papers at the Symposium on Diseases in Asian Aquacuture, 26-29 November, 1990. Of the 99 papers, 24 referred to *Vibrio* infections and 26 referred to *Aeromonas* infections. Virulent strains of *Aeromonas hydrophila* had been commonly isolated from fish with symptoms of epizootic ulcerative syndrome (EUS), a disease referred to by Prof. Roberts (University of Stirling) as "the new disease of the decade". We planned to develop diagnostic monoclonal antibodies to a virulent strain and screen a wide range of isolates held at the Australian Fish Health Reference Laboratory, CSIRO, Geelong and the University of Hokkaido, Hakodate, Japan. This work was planned to be completed by mid 1992.

Identification of fish eggs by monoclonal antibodies was the second part of the research. 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 (e.g. special pigmentation, size or disposition of internal features). Although the fish eggs of choice were tuna, about which little was known during early life-history stages, the first samples were lost in a CSIRO freezer malfunction and it has been impossible to obtain further samples due to research vessel schedules and costs. Snapper eggs were the next target for production of MAbs.

Summary of Results

(a) Diagnosis of Fish and Shell-fish Pathogens

Development of monoclonal antibodies against virulent strains of *Aeromonas hydrophila,* commonly isolated from fish with symptoms of epizootic ulcerative syndrome (EUS), was very successful. The results are documented in the attached manuscript which has been submitted to the Journal of Fish Diseases.

In brief, one monoclonal identifies the virulent strains of *A. hydrophila*, which also group as serotype I. The results were confirmed using two methods applied to screening live strains at the University of Hokkaido, Japan. Of particular interest was the screening of *A. hydrophila* strains, isolated from diseased fish in Australia, and now held at the Australian Fish Health Reference Laboratory, CSIRO, Geelong. Of 70 isolates tested, 10 were positive. Unfortunately, the virulence status of these positive strains is currently unknown, as is the origin of the strains. We are currently applying the diagnostics to new disease situations and continuing collaborative research with Australian and overseas laboratories.

(b) Identification of Fish Eggs

In regard to the development of monoclonal antibodies for identifying fish eggs some snapper eggs were obtained, but the quantity was insufficient to carry out antibody production and screening. Further supplies of snapper eggs were expected to become available but these did not eventuate due to lack of artificial spawnings.

We had already developed monoclonal antibodies against frozen eggs of *Lates calcarifer*, the barramundi, a species of commercial significance. Additional antibodies were developed against ethanol-fixed eggs. Screening of all antibodies was carried out using two indirect immunofluorescence assays, using anti-mouse FITC and anti-mouse rhodamine with eggs, as well as indirct ELISA and western blotting following SDS-PAGE. Fish eggs tested were from Barramundi, Blue-eye Trevalla, Mauhi (from Hawaii), Murray Cod and Snapper. Some autofluorescence was observed.

The sreening results were disappointing as the secondary conjugates reacted with the eggs, thus producing 'false-positives'. This means that the fish eggs possessed substances that reacted with the anti-mouse antibodies conjugated to fluorochromes or enzymes. Normally these anti-mouse antibodies would have only attached to the eggs coated with monoclonal antibodies as a secondary reaction.

The western blotting involved the separation of egg membranes and yolk by polyacrylamide gel electrophoresis (PAGE), which were then transferred onto nitrocellulose sheets for analysis using monoclonal antibodies, seconary anti-mouse HRP conjugate and subsequent colour development. It was obvious that the yolk components in particular reacted with the secondary anti-mouse conjugate but some membrane components also reacted. These data explained the disappointing 'false-negative' results.

Criteria for Evaluation

The project has been evaluated, and:
(a) monoclonal antibodies have been produced to identify virulent *Aeromonas hydrophila* strains, but not the target fish eggs due to wide cross-reactivities with eggs of other species,
(b) sufficient screening has been undertaken to confirm the specificity of the *Aeromonas hydrophila* antibodies,
(c) relevant user groups are aware of the existence of the techniques, and some have had the opportunity to collaborate in their use,
(d) the results have been submitted to the scientific literature, and
(e) 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 is occurring through the submission of a paper to the Journal of Fish Diseases (see attached manuscript) and presentation of the results to the forthcoming meetings of the Australian Mariculture Association and the 1993 Symposium on Diseases in Asian Aquaculture. We also plan to present a major paper to the 1993 meeting of 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

Transfer of technology in this form requires a joint venture with other parties (eg. CSL Ltd and Biotechnology Australia Ltd) to develop products. This is most relevant to the *Aeromonas* aspect of the current research and to the *Vibrio* aspects of the previous work.

Agreements for the market development of the *Aeromonas* and *Vibrio* diagnostics are presently being drawn up, jointly, with the Fisheries Research and Development Corporation and Deakin University.