FINAL REPORT

Title of the Project	**Monoclonal antibodies for identification and protection of fish and shell-fish against *Vibrio* pathogens**
Project Reference Number	92/67
Research Organisation	Deakin University
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Funding Corporation	Fisheries Research and Development Corporation

(ii) Non-technical summary

Our use of novel antigens (i.e. lipopolysaccharide molecules, LPS) isolated from three pathogenic *Vibrio* species, viz *V. salmonicida*, *V. tubiashii* and *V. splendidus,* resulted in the production of hybridomas that produced either species-specific or genus-specific mAbs. These results confirmed our view that the LPS molecules, that we isolated for immunising mice, contained a common *Vibrio* antigen, and had potential as a vaccine against Vibriosis.

All the mAbs produced in this work reacted with live and heat-treated bacterial cells. In addition, the reactions were not affected if cells had been killed with formalin or sodium azide. These treatments provide a means for sending inactivated infectious cells to diagnostic testing laboratories, thereby overcoming the main quarantine problems with live infectious cells. The mAbs developed during this 1-year project will be added to the library of mAbs that we now have for use in disease diagnosis.

Another main component of the 1-year project was to determine whether the LPS molecules could be used as a vaccine to prevent vibriosis. A summary of the results follows:

- The first strain of *V. anguillarum* had little effect on fish in passage tests, therefore a new Mt Pleasant isolate was obtained and subsequently confirmed to be virulent in passages.
- The LPS fragment used as the immunising antigen was isolated, identified using immunoblotting and quantified.
- Fish groups were immunised with 0-10μg doses of LPS fragment, or whole cells, in PBS, with no mortalities. This was repeated a week later.
- When the fish were challenged with 10^5 cfu's of *V. anguillarum* the whole-cell inoculum conveyed the best protection and the dose of 10μg LPS conveyed some protection. Results of using doses lower than 10μg LPS were similar to controls.
- Unfortunately, the trials in fish were not as clear cut as those done in mice. It is thought that the estimated dose of 10μg may have been substantially less due to aggregation of molecules and adsorption to containers prior to dilution of the sample to prepare inoculums and in the syringes.

The trial did show some protection by the LPS to the disease vibriosis, caused by *V. anguillarum*. It also emphasised the importance of testing the virulence of laboratory-held bacterial isolates, many of which had lost virulence through repeated in vitro culture. We plan to repeat the trial, using the virulent strain of *V. anguillarum* and with greater control on the quantity of immunising antigen.

(iii) Background to the Research

Diagnosis of Vibrio *and other Pathogens*

Our past research, funded by FIRDC, has resulted in the production of a World-class library of diagnostic monoclonal antibodies for fish and shell-fish pathogens, within the genera *Aeromonas* and *Vibrio* (Family Vibrionaceae). We have already developed diagnostic monoclonal antibodies against 10 pathogenic species of *Vibrio* (FIRTA GRANT 86/120; J. Fish Diseases, 15, 63-69, 1992 and J. Fish Diseases, 15, 331-337, 1992). In addition, mAbs have been developed during 1990/2 to identify pathogenic *Aeromonas* species, *A. salmonicida* and *A. hydrophila*. *A. salmonicida* is the causative agent of goldfish ulcer disease (GUD) that exists in Australia in both cultured and wild stocks of goldfish, and whereas *A. hydropila* is 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 have published the results of this research in the J. Fish Dis., 17, 123-133 (1994).

There were additional *Vibrio* species to which mAbs needed to be developed. *Vibrio salmonicida* is the causative agent of cold-water vibriosis in salmonids and cod (Sorum *et al.*, Appl. Environ. Microbiol. 56, 1990), whereas *V. tubiashii* and *V. splendidus* are pathogens of fish and shellfish (Myhr *et al.*, Appl. Environ. Microbiol. 57, 1991). Dr David Sutton of the Sir George Fisher Centre for Tropical Marine Studies has indicated problems in diagnosing these Vibrios. The plan was to use killed strains of virulent *V. salmonicida*, *V. tubiashii* and *V. splendidus* to produce monoclonal antibodies for diagnostic, aetiological and pathogenicity purposes.

Possible development of a single vaccine for all Vibrios

Production of fish vaccines for protection against pathogenic microorganisms has typically utilised the challenging of young fish with killed inoculums of virulent strains. Although this approach has had some success at development of protection against the virulent strain it usually conveys little, if any, protection against a range of related pathogens.

We had evidence that there is a common antigenic component on the surface of all *Vibrio* species, including all strains within each species. This arose from our findings that purified components of the bacterial cell wall could produce in mice a monoclonal antibody that reacted with all vibrios. A preliminary study had shown that the antigen was probably heat-stable lipopolysaccharide (LPS). Isolation of the common antigenic component would provide for preliminary testing of it as a vaccine in protecting against infection by any strain of *Vibrio*. Positive results would lead to considerable benefits in a wide range of aquaculture activities, especially in preventing the disease vibriosis.

(iv) Objectives of the Research Project

The objectives were to:
1. produce new monoclonal antibodies against strains of *V. salmonicida*, *V. tubiashii* and *V. splendidus*, for management of a range of problems in Australian fisheries and aquaculture, and to specifically utilise them and others already developed in detection, monitoring and control programs,
2. extensively test several mAbs that appear to recognise all *Vibrio* species and then isolate the common surface antigen, thereby providing for the testing of a single vaccine for all vibrios, and
3. continued collaborative research through provision of monoclonal antibodies or diagnostic services to programs associated with fisheries management.

(v) Introductory Technical Information

General Details of Monoclonal Antibody Technology

When an animal such as a mouse is injected with foreign chemicals (=antigens) such as bacteria, the usual response is a proliferation of a number of different antibody-producing cell clones, each one of which is committed to the production of a single antibody. Each antibody reacts with a specific entity (=epitope) on the injected material. Antibody producing spleen cells are then removed from the immunised mouse and fused (hybridised) to a mouse myeloma cell line (=cancer cells). Spleen cells and cancer cells, but not hybrid cells soon die in selective culture medium (=HAT medium) whereas hybrid cells grow and are assayed (=screened) for antibody production. Two assays used for identification of antibody production are the Enzyme-Linked Immunosorbent Assay (=ELISA) and fluorescein isothiocyanate (=FITC) immunofluorescence microscopy. Recloning and further assaying allows isolation of single clones (=monoclones), each producing a specific antibody (=monoclonal antibody). Desired clones, which react specifically with the material originally injected into mice, can be frozen for later use, when antibody supplies require replenishing. High yielding amounts of monoclonal antibodies can be obtained by injecting cloned cells into mice which develop tumours of the cloned cells and the ascites fluid of tumour-bearing animals can be up to 1000 times more concentrated than in culture medium. Various techniques exist for the purification of the monoclonal antibodies.

(vi) The Research Methodology

Production of Diagnostic Monoclonal Antibodies

Monoclonal antibodies were developed against LPS fragments isolated from virulent Type-strains of *V. salmonicida*, *V. tubiashii* and *V. splendidus* obtained from the American Type Culture Collection, Dr D. Sutton (Sir George Fisher Centre for Tropical Marine Studies, James Cook University of North Queensland) and Dr R. Wiik (Institute of

Marine Research, Bergen, Norway). They were then used in cross-screening tests to determine specificity.

To develop the mAbs, separate female Balb/c mice were given three immunisations of 0.2μg of LPS fragment at weekly intervals. Three days after the final injection spleen cells were removed and cell fusions made with mouse myeloma cells using polyethylene glycol (PEG-4000). Growth of hybrid cells were carried out in HAT culture medium (RPMI-1640 medium containing 10% V/V foetal calf serum plus hypoxanthine, aminopterin and thymidine = HAT). Hybrid clones producing mAbs reactive with *V. salmonicida*, *V. tubiashii* and *V. splendidus* strains were identified by ELISA, using whole cells. Positive clones were recloned, isotyped and stored frozen. High yielding amounts of mAbs were obtained by injecting appropriate cloned cells into mice, allowing tumours to develop and then collecting ascites fluid with 18 gauge needles. The mAbs were then stored at 4°C with sodium azide added. Additional screening for determining mAb specificity involved numerous ELISA tests using other *Vibrio* species and a wide range of gram-negative bacteria.

Testing of an LPS Fragment as a Vaccine

For the details of the methodology and results please refer to Appendix 2. In summary the methodology will follow the following 4 steps.

1. Preparation of an LPS extract from a virulent strain of *V. anguillarum* to act as the immunogen.
2. Preparation of challenge isolate, including culture of a virulent immunising strain of *V. anguillarum*. A biochemical profile of the challenge isolate will be checked and its activity verified in a Macroscopic Agglutination Test (MAT). Challenge isolate to be passaged through fish, three times, prior to aliquoting and storage of stock suspensions. Salmonid species with the highest sensitivity to *V. anguillarum* will be used (rainbow trout/Atlantic salmon). Approx. 10 fish required.
3. Determination of optimal challenge dose in which the lowest dose that will produce morbidity in 10 fish within 2 weeks will be used. This will be achieved by randomly selecting 8 groups of 10 fish (either rainbow trout/Atlantic salmon and 10 fish per 100 litre tank with artificial freshwater salts added, at 18°C) and acclimatising them for a minimum of 2 weeks prior to inoculation. The groups of fish will be inoculated with 0 to 10^7 cfu/fish of challenge strain suspended in 0.1 mL PBS. Fish that become moribund or die will be sampled and cultured (kidney and spleen) to verify cause of death. Isolates of *V. anguillarum* will be identified by minimal biochemical tests and MAT.
4. Immunisation with LPS fragment and challenge to determine whether LPS fragment is protective against *V. anguillarum*. 2 lots of 7 groups of 12 fish will be vaccinated IP with 0, 0.1, 0.3, 1, 3, 10 μg of LPS fragment per fish in 0.1 mL PBS, including 2 positive control (killed whole-cells) groups. Fish will be vaccinated at day 0 and day 7. Lot 1 will be challenged IP with a dose determined in part 2 above, at day 30. If protection is successful after day 30, fish in Lot 2 will be used to determine levels of serum antibody. If protection is not observed in Lot 1, Lot 2 will be boosted again and

challenged at day 60. Fish that become moribund or die will be sampled and cultured (kidney and spleen) to verify cause of death. Isolates of *V. anguillarum* will be identified by minimal biochemical tests and MAT.

A positive result would be shown by immunised fish being protected against vibriosis caused by *V. anguillarum*.

Continued Collaborative Research

We are committed to maintain, frozen in liquid nitrogen, the hybridomas developed during previous funding, and new ones as they are developed. When further diagnostic antibodies were required, we thawed and grew the hybidomas to produce adequate supplies of the antibodies. For example, antibodies used to screen a wide range of *A. hydrophila* isolates held at the Australian Fish Health Reference Laboratory, CSIRO, Geelong, and the University of Hokkaido, Hakodate, Japan.

(vii) Detailed Results and Discussion

Monoclonal antibodies have been produced to identify the target fish pathogens.

We successfully produced mAbs that reacted specifically with the bacterial strains *V. salmonicida*, *V. tubiashii* and *V. splendidus*. Screening of the mAbs against a wide range of gram-negative bacteria indicated that the mAbs specificities were as tabulated in Appendix 1. In particular, the 3 species-specific mAbs only react with one species, thus we have diagnostic mAbs for *V. salmonicida*, *V. tubiashii* and *V. splendidus*. However, it is unknown whether every strain of the species is reactive and only with long-term extensive testing, in the rapid detection in fish-health monitoring and control programs, will this be known. Two mAbs reacted with all *Vibrio* species and add to the growing number that we now have for diagnosing vibrios

Results of the production of diagnostic monoclonals were presented together with other work on pathogenic *Vibrio* species, to the 1993 meeting of the Australian Marine Sciences Association in Melbourne. Notification of results of our research has also occurred through the publication of papers in scientific journals (see attachments).

Testing of an LPS Fragment as a Vaccine

In the preliminary vaccine trials using the antigen from Vibrios we used groups of mice as they illicit a rapid immune responses which can be easily monitored by measuring antiserum titres. The mice immunised *i.m.* were found to develop a clear protection to oral infection by the same species of *Vibrio* and also showed a clear cross-protection to infection by another *Vibrio* species. Having obtained these excellent results we proceeded with vaccine trials with fish, in which less is known about the immune responses.

The fish vaccine trials were done in collaboration with Dr Mark Crane and Nick Gudkovs at the AAHL, CSIRO, whilst the Deakin University facilities were being modified. Small rainbow trout averaging 10.4cm and 14.0g were obtained from the Snob's Creek hatchery, randomly divided into groups and held in standard aquarium conditions. The details and results of the trial, using a cell-wall LPS fragment as an antigen to immunise the fish and a virulent strain of *V. anguillarum* as a challenge, are given in Appendix 2.

In particular, the trial did show some protection by the LPS antigen to the disease vibriosis, caused by *V. anguillarum.* It also showed the need for testing virulence of laboratory-held bacterial isolates, many of which lose virulence through repeated in vitro culture. The plan is to repeat the trial, using the virulent strain of *V. anguillarum,* and with greater control on the quantity of immunising antigen.

Continued Collaboration with Relevant User Groups

An increasing number of user groups have become aware of the results and are wanting to utilise the mAbs. We are continually assisting in the provision of our diagnostic mAbs and are assisting with immunodiagnostic testing. Examples include:

1. transfer of research quantities of some mAbs to the Tropical Health Program at the University of Queensland for use in water quality testing,
2. testing of bacteria causing diseases of shrimp in Tamil Nadu, India (Note: specimen obtained in formalin, from the Dean of the Veterinary and Animal Sciences University under AQIS permit),
3. testing of a formalin-fixed sample of bacteria isolated from diseased prawns in Thailand,
4. collaborative testing of mAbs to discriminate between strains of *V. anguillarum* and *V. ordalii* at the Department of General & Marine Microbiology, Goteborg University, Sweden,
5. testing of diseased prawns from a main hatchery in Queensland, and
6. testing of water, oyster larvae and scallop larvae from NSW hatcheries.

(vii) Discussion of Results

The results of the project have be evaluated according to whether:

1. new monoclonal antibodies have been produced to identify the target fish pathogens strains of *V. salmonicida*, *V. tubiashii* and *V. splendidus*,

We have developed new mAbs that add to the library of diagnostic mAbs already developed.

2. sufficient screening has been undertaken to confirm the specificity of the antibodies, and to specifically utilise them and others already developed in detection, monitoring and control programs,

As mentioned previously, some of these new mAbs have shown that they react specifically with the target species in screening tests carried out to date, but additional screening will be required to confirm specificity. The new mAbs, together with the others developed so far, make an impressive library of diagnostics that is currently being reviewed for commercial sales.

3. a common antigen has be identified in vibrios and shown to produce an immune response that protects against infection by vibrios, and

A common antigen has been identified for vibrios and this was confirmed in the use of the antigen to produce genus-specific mAbs. The use of the antigen to illicit protection against infection by vibrios was shown in a short mice trial. In a large trial in which rainbow trout were immunised with the antigen there appeared to be some protection given infection against by *V. anguillarum*. However, there was some uncertainty in the biochemical properties of the antigen in solution and thus the quantity used in immunising the trout. This vaccine trial will be repeated at Deakin University following a similar trial to protect fish against furunculosis caused by *Aeromonas salmonicida*.

4. relevant user groups are aware of the existence of the results, and at least some have had the opportunity to collaborate in their use

We are increasingly being contacted for supply of diagnostic mAbs and the technical knowledge to carry out the appropriate tests. It is expected that the mAbs will soon become available commercially. We will continue to do assist user groups with the proper use of the mAbs and develop more as the demands arise.

(ix) Implications and Recommendations

The costs and benefits of the 1-year project fall into 2 main categories.

Firstly, the production of mAbs has increasingly benefited Australian fisheries, and particularly aquaculture. As mentioned in an earlier report to FRDC we were instrumental in saving a major prawn hatchery in Queensland from closing down, as we were able to identify type and source of pathogen responsible for killing the juvenile prawns. A full report on this, including cost savings, is being prepared by ourselves and Dr N. Preston of CSIRO, and the FRDC will be given a copy. Additional testing of samples from other sources, and hatcheries in NSW, have identified problem areas. It is difficult to give a cost savings benefit at this stage but it is likely to be over AUS$1.5 million.

Secondly, in regard to vaccine production it is too early to give a true assessment of the benefits and cost savings to fisheries. We will be in a better situation to evaluate this component of the research at the end of the current project.

(x) Description of Intellectual Property

Joint ownership of the *Aeromonas* and *Vibrio* diagnostics on a 50:50 basis has been drawn up between the Fisheries Research and Development Corporation and Deakin University. The transfer of technology to industry, via the development and marketing of products, is underway. TECRA diagnostics (a division of Biotech Australia, which is fully owned by Hoescht) is the company through which the mAbs in test kits and research quantities are most likely to occur. FRDC will be informed of the progress.

(xi) Technical Summary

The most important technical information developed as part of the research has been the additional mAbs developed against *Vibrio* species. The characteristics of these mAbs are summarised in the table given in Appendix 1.

APPENDIX 1

Assessment of newly Developed Monoclonal Antibodies in Reacting with *Vibrio* species

Monoclonal Antibody	Isotype	Heat stability (LPS/Protein)	Genus Specificity	Species Specificity	Comments
F35P312F	IgM/κ	S (LPS)		*V. salmonicida*	Good reaction
F36P35G	IgA/λ	S (LPS)	*Vibrio*		
F36P310B	IgM/κ	S (LPS)	*Vibrio*		
F36P310B9G	IgM/κ	S (LPS)		*V. splendidus*	Good reaction
F37P27B	IgA/λ	S (LPS)		*V. tubiashii*	Weak reaction

S, heat stable antigen being LPS

APPENDIX 2

Assessment of Performance of a *Vibrio anguillarum* LPS subunit as a Vaccine in Salmonids

Summary plan of the proposed experimental work

1. Preparation of challenge isolate

a. Locate and culture AFHRL stock of original immunising strain used in mAb production at Deakin (Desheng to supply details of isolate/strain number, if isolate not found or not viable Desheng to supply viable culture).

b. Biochemical profile of challenge isolate to be checked and activity in Macroscopic Agglutination Test (MAT) to be verified (Desheng to supply mAb).

c. Challenge isolate to be passaged through fish, three times, prior to aliquoting and storage of stock suspension. Salmonid species with the highest sensitivity to *V. anguillarum* will be used (rainbow trout/Atlantic salmon). Approx. 10 fish required.

2. Determination of Optimal Challenge Dose

Determination of lowest dose that will produce morbidity in 10 fish within 2 weeks.

a. 8 groups of 10 fish (either rainbow trout/Atlantic salmon) will be acclimatised for a minimum of 2 weeks prior to inoculation. 10 fish per 100 litre tank with artificial freshwater salts added, at 18°C. Approx. 80 fish required.

b. Groups of fish will be inoculated with 0 to 10^7 cfu/fish of challenge strain suspended in 0.1 mL PBS.

c. Fish that become moribund or die will be sampled and cultured (kidney and spleen) to verify cause of death. Isolates of *V. anguillarum* will be identified by minimal biochemical tests and MAT.

3. Immunisation and Challenge

Titration of vaccine (LPS fragment) against challenge dose of bacteria. To determine if LPS fragment is protective against a challenge by *V. anguillarum* in rainbow trout/Atlantic salmon.

a. 2 lots of 7 groups of 12 fish will be vaccinated IP with 0, 0.1, 0.3, 1, 3, 10 µg of LPS fragment per fish in 0.1 mL PBS including 2 positive control (killed vaccine

by immersion) groups. Approx. 180 fish required (Desheng to provide 4 lots of 144 µg LPS fragment).

b. Fish will be vaccinated at day 0 and day 7. Lot 1 will be challenged IP with a dose determined in part 2 above, at day 30.
If protection is successful after day 30, fish in Lot 2 will be used to determine levels of serum antibody.
If protection is not observed in Lot 1, Lot 2 will be boosted again and challenged at day 60.

c. Fish that become moribund or die will be sampled and cultured (kidney and spleen) to verify cause of death. Isolates of *V. anguillarum* will be identified by minimal biochemical tests and MAT.

Deakin to Supply

Isolate number of original immunising strain and strain if not available at AFHRL.
MAb to original strain
LPS fragment, approx. 1 mg
Experimental fish, bacteriological media, syringes, hypodermic needles, sterile containers, aquarium supplies, water quality test kits, laboratory consumables.

AAHL to Supply

Isolates
Laboratory and aquarium facilities
Assistance to carry out laboratory work and maintain experimental fish.

Note included after meeting

Should include extra group in vaccination trial consisting of fish which have been immunised using formalin-killed whole cells of the challenge strain. Positive controls with formalin-killed homologous strain, by immersion.

***V. anguillarum* MAb's and LPS Samples - Preparation and Storage**

a. Prepared by Dr. Desheng Chen.

b. LPS sample labelled VAN01, AFHRL, LPS Frag, 1.08 gram (as a lyophilized powder in a 1/4 oz. bijou bottle). Stored at 4°C.

c. MAb samples TCSN F13P13F approximately 8 mL. Anti-*Vibrio anguillarum* VAN01, specific for serotype 01.

d. TCSN F14P55D 4/11/91 approximately 7.5 mL. Anti-*Vibrio anguillarum* VAN43, specific for serotype 01.

e. Ascites F11P411F 8/11/91 approximately 300µL. Anti-*Vibrio furnissi*, genus-specific for *Vibrio* (except *V. damsela*, which may not be a *Vibrio*)

f. LPS fragment. Add 1.080 mL of Milli-Q high purity water to stored sample. Swirl gently to resuspend. After cleaning sonicator probe in acetone and distilled water, sonicate LPS solution for 5 seconds.

g. Dispense 3 X 300µL, each containing 300µg (0.3 mg) of LPS, aliquots in sterile Sarstedt tubes and store at -80°C. The remaining 180µL was prepared for SDS-PAGE analysis.

h. LPS sample. Whole LPS in sample buffer, approximately 50µL. Load approx. 20µL/lane for electrophoresis.

LPS concentrations required for vaccination:

LPS per fish (µg)	Vol. of 1 mg/mL stock solution required (µL)	Volume of sterile PBSA (µL)	Total Volume (µL)
0	0	1500	1500
0.1	1.5	1498.5	1500
0.3	4.5	1495.5	1500
1	15	1485	1500
3	45	1455	1500
10	150	1350	1500

Volume of 1 mg/mL stock required 432µL

BACTERIAL CULTURE, PREPARATION OF INOCULA AND FISH PASSAGE

Bacterial strains

Vibrio anguillarum VAN01 ex Deakin Uni. Formerly AFHRL lyophilized bacterial culture collection No. AFHRL 2 isolate FD2. *V. anguillarum* Mt. Pleasant 85/3954-1, ex J. Carson.

Bacterial culture

VAN01 cultured 17/5, 48 hour culture on SBA at 24°C in air.

Harvested into PBSA using a bacteriological loop, absorbance read using a disposable 1 cm cuvette at 625 nm. 0.011

Made 10 fold serial dilutions to 10^{-7} and cultured, in triplicate, 50µL 10^{-3} to 10^{-7} on SBA at 26°C in air.

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	44	2	0	0
	TNTC	45	7	1	0
	TNTC	43	3	0	0
Average count	TNTC	44	4	0.33	0

O.D. 0.011 at 625 nm = 8.8 X 10^6 cfu/mL

Standardisation of bacterial suspensions. VAN01 cultured 19/5, 24 hour culture on SBA at 24°C in air. Harvested 5 suspensions into PBSA using a bacteriological loop, absorbance read using a disposable 1 cm cuvette at 625 nm. Made 10 fold serial dilutions to 10^{-7} and cultured, in triplicate, 50µL 10^{-3} to 10^{-7} on SBA at 26°C in air.

Sample 1

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	41	4	1
	TNTC	TNTC	53	5	0
	TNTC	TNTC	45	5	0
Average count	TNTC	TNTC	46.3	4.6	0.3

O.D. 0.025 at 625 nm = 9.2 X 10^7 cfu/mL

Sample 2

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	51	10	2
	TNTC	TNTC	62	0	0
	TNTC	TNTC	45	4	1
Average count	TNTC	TNTC	52.6	4.6	1

O.D. 0.027 at 625 nm = 1.06 X 10^8 cfu/mL. (This result was used as the basis for making a 10^7 per 100µL solution)

Preparation of inoculum for first fish passage

VAN01 cultured 21/5, 72 hour culture on SBA at 24°C in air.

Harvested into PBSA using a bacteriological loop, absorbance adjusted to 0.027 using a disposable 1 cm cuvette at 625 nm. From previous data on younger cultures this should yeild approximately 10^7 cfu/100µL. Three ten fold dilutions were then prepared to yield 10^4, 10^5, 10^6 and 10^7 cfu's/100µL. Fish were anaesthetised with 50mg/l benzocaine and inoculated intraperitoneally on the left side with 100µL of each suspension. Serial

dilutions of the 10^7 solution were then prepared and 50µL cultured in triplicate to check purity and size of inoculum.

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	155	21	0	1	0
	159	24	3	0	0
	157	23	3	0	0
Average count	157	22.6	2	0.3	0

O.D. 0.027 at 625nm = 4.52 X 10^6 cfu/mL.

Standardisation of bacterial suspensions

VAN01 cultured 24/5, 24 hour culture on SBA at 24°C in air. Harvested into PBSA using a bacteriological loop, absorbances measured using a disposable 1 cm cuvette at 625 nm.

Sample 1

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	28	2	0
	TNTC	TNTC	27	1	0
	TNTC	TNTC	31	3	0
Average count			28.6	2	0

O.D. 0.028 at 625nm = 5.72 X 10^7 cfu/mL

Sample 2

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	51	7	0
	TNTC	TNTC	41	2	0
	TNTC	TNTC	49	4	0
Average count			47	4.3	0

O.D. 0.031 at 625nm = 9.4 X 10^7 cfu/mL

Sample 3

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	53	10	2
	TNTC	TNTC	62	11	2
	TNTC	TNTC	54	12	2
Average count			56.3	11	2

O.D. 0.028 at 625nm = 1.126 X 10^8 cfu/mL

Sample 4

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	64	11	0
	TNTC	TNTC	58	13	2
	TNTC	TNTC	57	11	4
Average count			59.6	11.6	2

O.D. 0.027 at 625nm = 1.192×10^8 cfu/mL

Sample 5

Plate counts (cfu)	010^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	60	5	0
	TNTC	TNTC	68	11	0
	TNTC	TNTC	58	13	2
Average count			62	9.6	0.6

O.D. 0.030 at 625nm = 1.24×10^8 cfu/mL

Virulent Tasmainian Isolate: *V. anguillarum* Mt. Pleasant 85/3954-1, ex J. Carson

Preparation of inoculum using Tasmanian isolate 85/3954-1 and inoculation of RT.

Sample 6

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	70	11	0
	TNTC	TNTC	75	2	1
	TNTC	TNTC	73	12	2
Average count			72.6	8.3	1

O.D. 0.047 at 625nm = 1.45×10^8 cfu/mL in original sample.

Passage

Fish 13A&B Inoculated with 100µL of a 1:10 dilution, 1.45×10^6 cfu (low dose).

Fish 14A&B Inoculated with 100µL undiluted, 1.45×10^7 cfu (high dose).

Day 2 Fish in both groups displayed abnormal swimming behaviour, dark in colour.

Day 3 13A and 14A & B dead. Kidney and spleen from all fish cultured on HBA, at 25°C in air. Samples of kid. and spl. were also taken and frozen at -80°C.

Day 4 All cultures from 15/7 yeilded a heavy pure growth of *V. anguillarum*. Growth from these plates was harvested into TSB, 10% glycerol and stored frozen at -80°C.

Inoculation of fish with kidney tissue from Day 3

A tissue homogenate consisting of approximately 50% thawed infected kidney tissue and 50% sterile PBSA was passed through a 21G hypodermic needle.

Day 1 Fish 14C was anaesthetised and inoculated with 500µL of the suspension using a 19 G needle.

Day 3 Fish 15A found dead, kidney and spleen were cultured and Gram stained. Tissues frozen at -80°C.

Day 4 Cultures from 15A yielded heavy pure growth of *V. anguillarum*. Bacteria, passaged through fish twice, were harvested from this culture and frozen at -80°C for future experiments.

V. ANGUILLARUM FISH INOCULATION EXPERIMENT

Aim To determine the optimal dose of *V. anguillarum* for challenge of rainbow trout in LPS-fragment vaccination trial.

Fish The fish used in this experiment were rainbow trout from Snobs Creek Hatchery. These were the remaining fish from the first batch brought into AAHL, LAF Room C7 under Experimental protocol number OA2/7/2/ 355.

Bacteria *Vibrio anguillarum* Mt. Pleasant 85/3954-1, passaged twice.

Preparation of Inoculum

The Mt. Pleasant isolate was passaged twice through fish. A 24hr culture (HBA at 22°C in air) of kidney tissue from the second passage fish with Vibriosis was used to prepare the inoculum. A small amount of culture was suspended in sterile PBSA to yield an absorbance of 0.040 at 625 nm. Serial ten-fold dilutions of this suspension were prepared and used as inoculum. The purity, viability and concentration of viable cells in the inocula was determined by serial dilution and culture.

Plate counts (cfu)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	54	5	0
	TNTC	TNTC	54	2	0
Average count			54	2.3	0

O.D. 0.040 at 625nm = 1.08×10^8 cfu/mL (fish inoculated with 100µL)

Procedure

a. Fish were randomly selected and divided into 8 groups. The details of tanks numbers, fish numbers and dose are summarised in Table 1.

b. All fish were anaesthetised prior to inoculation with 100µL of PBSA containing bacteria (dose summarised in Table 1) using a 1 mL syringe and 23G needle. The fish were revived in fresh water and returned to their individual tanks.

c. Fish were observed daily and details of mortalities recorded (Table 2).

d. All dead fish were subjected to bacteriological examination in order to determine if the cause of death was Vibriosis.

Bacteriological examination of dead fish

All fish were cultured for *V. anguillarum*.

Fish which died during the course of the experiment were examined as follows:

a. The fork length and body weight was measured and recorded.

b. The presence of any gross external lesions was recorded.

c. The fish were then surface disinfected with 70% ethanol prior to dissection.

d. The presence of any gross internal lesion was recorded.

e. The kidney and spleen was cultured on SBA at 22°C in air. Smears for Gram staining were also prepared from these tissues. Samples of kidney and spleen were placed in sterile Sarstedt tubes and frozen at -80°C.

Results

Table 1. Fish Group and Inoculation Summary

Tank Number	Number of Fish	Dose (cfu)	Fish Numbers
1	5	PBSA Control	A 1-5
2	5	1.08×10^1	B 1-5
3	5	1.08×10^2	C 1-5
4	8	1.08×10^3	D 1-8
5	8	1.08×10^4	E 1-8
6	8	1.08×10^5	F 1-8
7	8	1.08×10^6	G 1-8
8	8	1.08×10^7	H 1-8

Table 2. Record of Mortalities

Tank	Group	Dose	Day 2	Day 3	Day 4	Day 5	Day 6
A	1	10^0					
B	2	10^1					
C	3	10^2					
D	4	10^3			5		1
E	5	10^4		1	7	1	
F	6	10^5		5	3		
G	7	10^6		8			
H	8	10^7	1	7			

Day 2

H1 43.9g, 16.3cm Few gross external lesions. The inoculation site was slightly raised and there was reddening and haemorrhage around vent. Internally there were small haem's over the surface of the internal organs and over the surface of lateral musculature. Day 3. Cultures of kidney and spleen yielded HPG of *V.anguillarum*. Bacteria were harvested for PAGE.

Day 3

E1 40.98g, 16.6cm No external lesions. Reddening and haemorrhaging around vent. Internally, there was slight haemorrhaging at the inoculation site, a reddening of the gut, and an enlarged spleen. Day 6 Kid. HG *V. ang*.

F1 86.31g, 19.5cm Externally, there was a reddening of the vent, a raised inoculation site, and a slight reddening of the gills. Internally, a reddening of the fish's fat could be seen, and the spleen was slightly enlarged. Day 6 Kid. HG *V. ang*.

F2 44.73g, 15.8cm Inoculation site was darkened, and vent was reddened. Internally, there was severe haemorrhaging, and the spleen had almost completely liquefied. Day 6 Kid.and Spl. HG *V. ang*.

F3 99.7g, 20.2cm Inoculation site was slightly raised. There was a small amount of blood at the vent. Internally, there was partial haemorrhaging at the inoculation site, and the kidney was partly liquefied. Day 6 Kid.and Spl. HG *V. ang*.

F4 87.4, 20.1cm Inoculation site was dark and raised, and the gills were also quite darker than usual. Internally, there was partial haemorrhaging at the

	inoculation site, and the kidney was partly liquefied. Day 6 Kid. and Spl. HPG *V. ang.*
F5 50.0g, 17cm	Inoculation site was raised, and the vent had haemorrhaged. Internally, there was a reddening of the gut, and the spleen was partially enlarged. Day 6 Kid. and Spl. HPG *V. ang.*
G1 51.94g, 17.3cm	Externally, there was a reddening of the vent. Internally, the organs were of yellow colouring, the spleen and kidney were of liquid formation, and the swim bladder had been destroyed. Day 6 Kid. and Spl.. HMG predomin. *V. ang.*
G2 94.57g, 20.5cm	Externally, there was a reddening of the vent. Internally, the organs were quite liquefied. Day 6 Kid. and Spl. HMG, predom. *V. ang.*
G3 65.58g, 18.8cm	Externally, there was a large lesion at the inoculation site, and there was also a reddening and haemorrhaging at the vent. Internally, the organs were of yellow colouring,and the spleen and kidney were of liquid formation. Day 6 Kid. HG *V. ang.*
G4 55.84g, 17.2cm	Inoculation site was darkened and slightly raised. Severe haemorrhaging of the vent. Internally, the organs were completely liquefied, but the kidney was not affected.
G5 64.33g, 16.9cm	There was a reddening of the vent externally, but internally, the organs were sampled for histology. Day 6 Kid. and Spl. HMG, few if any *V. ang.*
G6 42.29g, 15.9cm	Severe external lesions. Haemorrhaging and reddening at the vent, and one eye was missing. Internally, the organs were completely liquefied. Day 6 Kid. HG *V. ang.*
G7 66.50g, 18.7cm	Dark and raised inoculation site, and haemorrhaging at the vent. Internally, the kidney was partially liquefied. Day 6 Kid. HG *V. ang.*
G8 90.1g, 20.1cm	Haemorrhage at the vent. Lesion at inoculation site. Internal organs were sampled for histology.
H2 77.13g, 19.1cm	Raised inoculation site, and haemorrhaging at he vent. Internally, the organs were partially enlarged. Day 6 Kid. HMG incl. *V. ang.*
H3 54.10g, 17.8cm	Raised inoculation site, and haemorrhaging at he vent. Internally, the organs were partially enlarged. Day 6 Kid. HMG incl. *V. ang.*

H4 54.9g, 18.1cm	Lesion at the inoculation site, a reddening of the vent, and lesions in skin. Internally, the organs were completely liquefied, including the kidney. Day 6 Kid. HMG incl. *V. ang*.
H5 70.04g, 18.1cm	Raised inoculation site, and reddening of the vent. Internally, the organs were unidentifiable, but the kidney had not been affected. Day 6 Kid. HMG incl. *V. ang*.
H6 82.71g, 19.1cm	Lesions in skin and raised inoculation site. Internally, there was a haemorrhaging at the inoculation site, and the organs were partially enlarged. Day 6 Kid. HMG incl. *V. ang*.
H7 49.89g, 17.4cm	Raised inoculation site. Internally, all organs were liquefied, including the kidney. Day 6 Kid. HMG incl. *V. ang*.
H8 26.6g, 13.2cm	Raised inoculation site. Internally, all organs were liquefied, including the kidney. Day 6 Kid. HMG incl. *V. ang*.
Day 4	All cultures were made on HBA and incubated at 22°C in air.
D1 71.51g, 18.7cm	Raised inoculation site, reddening of the vent. Internally, there was no haemorrhaging, but the spleen was enlarged and partially liquefied. Day 6 Kid. & Spl. HPG *V. ang*.
D2 51.72g, 16.8cm	Lesion of the inoculation site. Internally the organs were partly liquefied, and the spleen was missing. Day 6 Spl. HPG Kid. HG, predom. *V. ang*.
D3 85.77g, 19.1cm	Lesions in skin, a raised inoculation site, and reddening of the vent. Internally, there was complete reddening of the organs. Day 6 Kid. and Spl. HPG *V. ang*.
D4 44.51g, 16.2cm	Raised inoculation site and reddening of the vent. Internally, no tissues were able to be collected as the organs were so liquefied that they were unidentifiable.
D5 49.13g, 16.8cm	Reddening of the vent. Internally, the spleen was enlarged and there was severe haemorrhaging at the inoculation site. Day 6 Kid. HG *V. ang*.
E2 109.27g, 21.4cm	Severe lesion at the inoculation site, and vent was swollen and reddened. Internally, there was haemorrhaging at the inoculation site and the spleen was enlarged. Day 6 Kid. and Spl. HPG *V. ang*.

E3 54.98g, 16.6cm — Lesion at the inoculation site. Internally, the organs were discoloured, and there was haemorrhaging at the inoculation site. Day 6 Kid.and Spl. HG *V. ang.*

E4 92.4g, 19.6cm — Raised inoculation site and reddening of the vent. Internally, there was haemorrhaging at hte inoculation site. Day 6 Kid.and Spl. HPG *V. ang.*

E5 65.7g, 19.1cm — Severe lesion at inoculation site, reddening of vent, and skin was dark in places. Internally, there was haemorrhaging at the inoculation site. Day 6 Kid.and Spl. HPG *V. ang.*

E6 75.1g, 19.5cm — Raised inoculation site and reddening of the vent. Internally, there was severe haemorrhaging at the inoculation site and the gut was red and enlarged. Day 6 Kid.and Spl. HMG includ. *V. ang.*

E7 64.3g, 18.6cm — There was reddening of the vent externally, and internally, there was haemorrhaging at the inoculation site. Day 6 Kid.and Spl. HPG *V. ang.*

E8 63.7g, 17.9cm — Severe lesion at the inoculation site, and haemorrhage of the vent. Internally, there was severe haemorrhaging at the inoculation site. Day 6 Kid.and Spl. HMG predom. *V. ang.*

F6 82.4g, 19.8cm — Raised inoculation site, reddening of the vent, and reddening of the gills. Internally, the spleen and fat deposits were enlarged. Day 6 Kid.and Spl. HPG *V. ang.*

F7 56.96g, 17.7cm — Severe lesion at inoculation site. Internally, there was haemorrhaging at the inoculation site, the spleen was partially liquefied, and the swim bladder had been destroyed. Day 6 Kid.and Spl. HPG *V. ang.*

F8 61.76g, 17.4cm — Raised inoculation site and haemorrhaging of the vent. Internally, there was severe haemorrhaging of the inoculation site, and the organs were completely liquefied. Day 6 Kid.and Spl. HMG predom. *V. ang.*

Day 5

E9 54.23g, 16.3cm — Gross external lesions; enlarged and reddened vent; abdomen severely enlarged, possibly ascites?? Internally, there was severe haemorrhaging of all organs and the spleen was missing. There was also bile in the gut. Day 8 Kid. and Spl. HPG *V. ang.*

Day 6

D6 55.8g, 16.8cm	Slight haem externally at inoculation site. Internally enlarged spleen, kidney appeared normal. Day 8 Kid. and Spl. HPG *V. ang*.
Day 7	No mortalities
Day 8	No mortalities. Experiment terminated. Kidney of remaining fish cultured on SBA for *V. anguillarum*.
Day 9	Cultural results of surviving fish. LAF Daily record.

V. ANGUILLARUM FISH VACCINATION EXPERIMENT

Aim

To determine the ability of the LPS fragment derived from *V. anguillarum* to immunise against *V. anguillarum* infection.

Experimental Fish

a. The fish used were rainbow trout from Snobs Creek Hatchery. These were the second batch of smaller fish brought into the LAF under experimental protocol number 355. 40 fish were measured at an average of 10.4 cm, 14.0 g on arrival.

b. Fish were randomly selected and distributed in groups of 15 into 14 individual 150 L aquaria. Each aquarium contained R.O. water with "artificial freshwater salts" added at 18^{o}C and was supplied with a 150mm air lift biofilter. A small amount of feed in the form of floating pellets was offered to the fish, which began feeding in most tanks.

c. Fish were allowed to acclimatise for 7 days. 41 fish died the day after arrival and this was attributed to post transport stress as only 1 mortality was observed on the following day. There were no more losses of fish until another 5 days when 1 fish in tank 5 was found dead.

Feeding

Fish were fed 2.5% of average bodyweight per day. On weekdays feed was given morning and evening on the weekends fish were fed once in the morning. Feed per tank of 14 fish (196 g) 4.9 g per day (2 X 5 mL in 15 mL centrifuge tube)

Vaccination (Day 1 of the experiment)

Table 1. Tank No's. and LPS concentrations required for vaccination:

LPS per fish (µg)	Vol. of 1 mg/mL stock solution required (µL)	Volume of sterile PBSA (µL)	Total Volume (µL)	Tank No.
0	0	1500	1500	1 and 8
0.1	1.5	1498.5	1500	2 and 9
0.3	4.5	1495.5	1500	3 and 10
1	15	1485	1500	4 and 11
3	45	1455	1500	5 and 12
10	150	1350	1500	6 and 13
Whole cell vaccinated	0	0	0	7 and 14

Volume of 1 mg/mL stock required 432µL for 10 LPS vaccinated tanks.

Procedure

a. All fish were anaesthetised prior to vaccination with 100 µL of PBSA containing the LPS fragment (doses summarised in table 1) using a 1 mL syringe and 26G X 13 mm needle. The fish were revived in fresh water and returned to their individual tanks.
b. Fish were observed daily and details of any mortalities recorded.
c. All dead fish were subjected to bacteriological examination in order to determine the cause of death.

Results

Day 2 All fish feeding well, no mortalities.

Day 3 All fish feeding well, no mortalities.

Day 4-8 No mortalities, all fish feeding well, except T2 (slight bacterial bloom, 30% water change T2 on Day 6).

Day 8 The vaccination procedure from Day 1 was repeated.

Day 18 Five fish found dead in Tank 5. Length and weight was measured and recorded, with results as follows: Length = 10cm; Weight = 16.086g.

Day 26 One mortality in Tank 8.

Day 27 Nitrite levels were measured and recorded before water changes were completed. Results are as follows: Tank 1 - 10ppm, T2 - 5ppm, T3, 7,11,

13 and 14 were all above 20ppm, T4, 5, 6, 9,and 12 were at 20ppm, T8 and 10 were at 15ppm, and the 50 litre tank recorded 40ppm.

Day 28 Nitrite levels were measured and recorded after water changes were completed. Results are as follows: Tank 1, 4, 5, 8, 9, 10, 11, 12, and 13 all recorded 20ppm, T2 recorded 0ppm, T3, 6, 7, 14 and the 50L tank all recorded 10ppm. Ammonia tests were carried out on tanks 2, 7, 10 and 13. T2 and T13 recorded approximately 0.8ppm, T7 approximately 0.7, and T10 approximately 0.9ppm. Water changes of 30% were made to all tanks except T3 and T7, which were given 60%, and the 50L tank was given 10%. The top disks of filters in T2, 5, 6, 10, 11 and 12 were all cleaned, and the remaining disks left as they were.

Pilot Inoculation

This was prepared from a 24h culture of the isolate made on SBA at 22°C in air. The organism was suspended in sterile PBSA to an O.D. of 0.04 at 625nm. Serial dilution and culture confirmed this suspension to be 7.5 X 10^7 cfu/mL (was meant to be 1 X $10^{8)}$.

Plate counts (cfu)	10-3	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	TNTC	TNTC	TNTC	4	4
	TNTC	TNTC	42	6	0
	TNTC	TNTC	28	2	1
Average count			35	4	0.3

Day 35 3 groups of 3 fish were inoculated with 10^3 to 10^5 cfu's of the challenge isolate.
Tank A recieved 1:100 dilution containing 7.5 X 10^4 cfu
Tank B recieved 1:1,000 dilution containing 7.5 X 10^3 cfu
Tank C recieved 1:10,000 dilution containing 7.5 X 10^2 cfu

Day 36 No mortalities

Day 37 1 fish in group A dead. All dead fish were cultured, SBA.
3 fish in group B "
2 fish in group C "
Heavy pure growth of *V. anguillarum* from both kidney and spleen in all dead fish.

Day 38 2 remaining fish from group A dead.
Heavy pure growth of *V. anguillarum* from both kidney and spleen in all dead fish.

Day 41 Single live fish in group C terminated.

BACTERIAL CHALLENGE

Challenge Isolate *Vibrio anguillarum* Mt. Pleasant 85/3954-1

Preparation of Inoculum

The Mt. Pleasant isolate was passaged twice through fish. A 24h culture (HBA at 22°C in air) of the frozen broth from the second passage fish with Vibriosis was used to prepare the inoculum. A small amount of this culture was suspended in sterile PBSA to yield an absorbance of 0.040 at 625 nm. Previous experiments have shown that this should yield approximately 1 X 10^8 cfu/mL.

Inoculation (Day 41 following 1st vaccination)

At this stage fish had an average weight of 28.77g and length of 13.12cm (n=37). Fish were inoculated at 14g and 10.4cm.

a. Groups of 10 fish were used for each treatment.
Tanks 1-7 inclusive received the "high" challenge dose (10^{-2} dilution of 0.04 suspension) approximately 10^5 cfu's per fish, tanks 8-14 received the low dose (10^{-3} dilution of 0.04 suspension) approximately 10^4 cfu's per fish. The actual dose was determined from cultures of serially diluted inoculum. In this case, the O.D.=0.04 suspension used to prepare the inoculum was serially diluted and cultured and the inocula themselves were also serially diluted and cultured.

Plate counts for 50µL of ten fold dilutions of 0.04 suspension.

	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Plate 1	TNTC	TNTC	45	4	0
Plate 2	TNTC	TNTC	44	4	1
Plate 3	TNTC	TNTC	39	6	1
Average			42.6	4.6	0.6

	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Plate 1	TNTC	TNTC	33	3	0
Plate 2	TNTC	TNTC	27	7	1
Plate 3	TNTC	TNTC	36	4	3
Average			32	4.6	1.3
Average of 2 estimations			37.3	4.6	

Using 37.3 cfu's at 10^{-5} dilution
37.3 X 20 cfu/mL at 10^{-5}
37.3 X 20 X 10^5 cfu/mL of 0.04 suspension.
7.46 X 10^7 cfu's/mL

Both 'high' and 'low' dose inocula were cultured to check purity and viability. Both cultures were pure and viable.

b. All fish were anaesthetised prior to inoculation with 100µL of PBSA containing bacteria (dose summarised in table 2) using a 1 mL syringe and 26G needle. The fish were revived in fresh water and returned to their individual tanks.

c. Fish were observed daily and details of mortalities recorded.

d. All dead fish were subjected to bacteriological examination in order to determine if the cause of death was Vibriosis.

Sampling and bacteriological examination of dead fish

Fish which died during the course of the experiment were examined as follows:

a. The presence and appearance of any gross external lesions was recorded.

b. The fish were surface disinfected with 70% ethanol prior to dissection.

c. The presence of any gross internal lesion was recorded.

d. The kidney and spleen was cultured on SBA at 22°C in air. Smears for Gram staining were also prepared from these tissues. Samples of kidney and spleen were stored in sterile Sarstedt tubes at -80°C.

e. Samples were taken for histology.

Record of mortalities following challenge on Day 1

Tank	Day 2	Day 3 AM	Day 3 PM	Day 4 AM	Day 4 PM	Day 5 AM	Day 5 PM	Day 6 PM	Day 8 AM	Day 9 AM
1	1/1	8/9	0/9	0/9	1/10	-	-	-	-	-
2	1/1	4/5	3/8	2/10	-	-	-	-	-	-
3	1/1	6/7	1/8	2/10	-	-	-	-	-	-
5	1/1	7/8	1/9	1/10	-	-	-	-	-	-
6	3/3	4/7	1/8	1/9	0/9	1/10	-	-	-	-
7	0/0	0/0	0/0	2/2	0/2	0/2	0/2	0/2	0/2	0/2
8	0/0	5/5	2/7	1/8	0/8	0/8	0/8	0/8	0/8	0/8
9	0/0	5/5	1/6	3/9	0/9	0/9	0/9	0/9	0/9	0/9
10	0/0	3/3	1/4	4/8	0/8	0/8	0/8	1/9	0/9	0/9
11	0/0	4/4	2/6	2/8	1/9	0/9	0/9	0/9	0/9	0/9
12	0/0	9/9	1/10	-	-	-	-	-	-	-
13	0/0	2/2	2/4	2/6	0/6	0/6	0/6	0/6	0/6	0/6
14	0/0	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1

General observations and cultural results

Day 1 Fish were challenged AM.

Tank No.	Estimated dose (cfu's)	Calculated Dose (cfu/fish)	No. of fish per group	Inoculum Volume (µL)
1-7 (no tank 4)	10^5	7.46 X 10^4	10	100
8-14	10^4	7.46 X 10^3	10	100

Day 2 AM - Fish in tanks 1,5,8,10,13, and 15 not feeding well. Uneaten food in T1,T5 and T8. PM - Fish in T1,5, and 8 not feeding well. Fish began to die. Dead fish were stored at 4°C overnight before culture.

Day 3 AM - All tanks not feeding, except T7, T13, T12 and T14.

Day 4 All fish cultured from Day 2 and 3 showed HPG of *V. anguillarum*, except culture 1 from tank 6 Day 3 (spleen) from which there was no growth.

Day 5 All fish cultured from Day 4 displayed HPG of *V. anguillarum*. An isolate from T11 was frozen and stored at -80°C for future reference.

Day 8 Culture from Day 4 T1 and T11 and Day 5 T6 all displayed HPG of *V. anguillarum*.

Day 9 No cultures to examine. Experiment terminated as there had been no mortalities for 4 days. All remaining fish were bled. Serum sample numbers were as follows: T7 8 samples, T8 2, T9 1, T10 1, T11 1, T13 4, T14 9.

The spleens from 2 fish from T14 were cultured to ensure elimination of *V.anguillarum* from the surviving fish.

Tissues for histology

Day 3 Dead challenged fish - visera, kidney with adherant muscle and head were taken from 3 fish from T2 and 2 fish from T11. These fish died during the day, between the morning and afternoon sampling and were very fresh.

Day 9 Live fish surviving challenge - head, kidney section with adherent muscle and viscera were taken from T11 1 fish, T13 2 fish, T14 2 fish. 2 whole fish from T14 were fixed whole in 10% NBF and 2 other whole fish from T14 were fixed whole in bouin's solution for comparison.

Gross Lesions

During the course of the experiment there was a rapid devopment of bacterial septiceamia in the challenged fish. Early mortalities 24-48h were characterised by haemorrhage at the base of fins, especially the pelvic and dorsal fins. There was reddening around the vent and in many cases this was accompanied by a mucoid discharge from the vent. After 72 h there was swelling and haemorrhage around the inoculation site and internally a marked increase in haemorrhage in the lateral musculature. Late mortalities from the low dose group showed haemorrhage over the visceral surface, adoema, reddening of the surface of the intestine and swelling and darkening of the spleen. In some cases the spleen was completely liquefied.

COMPARISON OF *VIBRIO ANGUILLARUM* ISOLATES FOR VACCINATION EXPERIMENT

Isolate summary

VAN01 ex Deakin University, formerly FD2 (lyophilized culture No. AFHRL 2)
Vibrio anguillarum BAC_NSF 317
Vibrio anguillarum Fish Vibrio 7/80 (lyophilized culture No. AFHRL 2)
Vibrio anguillarum Fish Vib (lyophilized culture No. AFHRL 2)
Vibrio anguillarum BAC_NSF 32

MAb and LPS fragment preparation and storage

LPS sample labelled	VAN01, AFHRL, LPS Frag, 1.08 gram (as a lyophilized powder in a 1/4 oz. bijou bottle). Stored at 4°C.
MAb samples	TCSN F13P13F 8/11/91 approximately 8 mL TCSN F14P55D 4/11/91approximately 7.5 mL Ascites F11P411F 8/11/91 approximately 300µL

1. Add 1.080 mL of Milli-Q high purity water. Swirl gently to resuspend.

2. After cleaning sonicator probe in acetone and distilled water, sonicate LPS solution for 5 seconds.

3. Dispense 3 X 300µL, each containing 300µg (0.3 mg) of LPS, aliquots in sterile Sarstedt tubes and store at -80°C. The remaining 180µL was prepared for SDS-PAGE analysis.

MAb sample	TCSN F13P13F approximately 6 mL
LPS sample	Whole LPS in sample buffer, approximately 50µL. Load approx. 20µL/lane for electrophoresis.

PREPARATION OF LPS FOR SDS-PAGE

100µL of LPS 1mg/mL was added to 500µL of 1X mercaptoethanol buffer and heated at 100°C for 5 minutes.

Final concentration of LPS is approximately 0.1666 µg/µL.

SDS-PAGE AND WESTERN BLOT ANALYSIS OF ISOLATES AND LPS FRAGMENT.

Isolates prepared for SDS-PAGE by standard method for gram negative bacteria (m:\met\page\bac_page NG)

2 x 12% Gels, Laemlli Buffers

Gel 1 using unstained standards for Coomassie Blue
Gel 2 using prestained standards for Western blot transfer.

Lane	Sample	Vol (µL)
1	LMW Stds	0 5
2	LMW Stds	0 5
3	VAN01 ex Deakin	10
4	*V. anguillarum* BAC_NSF 317	10
5	*V. anguillarum* Fish Vibrio 7/80	10
6	*V. anguillarum* Fish Vib	10
7	*V. anguillarum* BAC_NSF 32	10
8	VAN01 LPS frag. (prepared 25/5/93)	10 (1.6µg LPS)
9	LMW Stds	0 5
10		

Western blot: (standard conditions, m:\met\page\wblot NG)
Transferred at 4°C for 1.5 hours at 100 volts.
Blocked O/N at 4°C, SM.
TCSN MAb F13P13F, used undiluted 2 hours at 37°C.
Silenus S anti Mouse,HRPO @ 1:500

Electrophoresis result

Relatively good balance of loading was achieved between the samples. The first four samples derived from isolates;
VAN01 ex Deakin University, formerly FD2 (lyophilized culture No. AFHRL 2)
V. ang BAC_NSF 317
V. anguillarum Fish *Vibrio* 7/80 (lyophilized culture No. AFHRL 2)
V. anguillarum Fish Vib (lyophilized culture No. AFHRL 2)

appeared to be identical. Sample 5 from isolate V. ang BAC_NSF 32 was very similar however the major band at approximately 45kDa had a significantly higher molecular weight, approximatley 47kDa. The LPS preparation showed no staining with Coomassie Blue as expected.

Western blot result

Very little activity was observed. The intensity of the single bands observed in some lanes was directly related to the initial loading. A band was observed at approximately 48kDa in decending order of intensity in lanes 4,7,3 and 5, no bands were detected in lanes 6 and 8 which corresponded to FD2 isolate "Fish Vib" and the LPS preparation. The western should be repeated with a higher loading of sample.

SDS-PAGE AND WESTERN BLOT ANALYSIS OF ISOLATES AND LPS FRAGMENT.

Same samples and conditions as before, however, 1.5 mm gels were used in order to achieve a higher sample loading.

Gel 1 using unstained standards for Coomassie Blue
Gel 2 using prestained standards for Western blot transfer.

Lane	Sample	Vol (µL)
1	LMW Stds	5
2	LMW Stds	5
3	VAN01 ex Deakin	50
4	*V. anguillarum* BAC_NSF 317	40
5	*V. anguillarum* Fish Vibrio 7/80	50
6	*V. anguillarum* Fish Vib	70
7	*V. anguillarum* BAC_NSF 32	60
8	VAN01 LPS frag. (prepared 25/5/93)	60 (9.6µg LPS)
9	LMW Stds	5
10		

Western blot: (standard conditions, m:\met\page\wblot NG)
Transferred on ice O/N for approximately 18 hours at 10 volts.
TCSN MAb F13P13F 8/11/91, ex Desheng (Deakin) undiluted 2 hours at 37°C.
Silenus S anti Mouse,HRPO @ 1:500

Electrophoresis result

Resolution was extremely poor in all lanes except 5 which was acceptable. Two factors may have contributed to this, the first and most likely being too much protein loaded. The second may have been the electrophoresis itself, a lot of heat was generated in the thicker gels run at 200V, gels and buffer were quite warm at the end of the run. Significant

amounts of protein were present in all lanes, except 8, which contained the LPS sample. A very mall amount of staining was observed in lane 8, probably due to spill over from lane 7 during loading. The 1.5 mm ten well comb was at its limit when taking around 60-70µL of sample.

Western blot result

Transfer of prestained standards was very good. These results were unexpected given the results from the previous experiment. With the increased loading significant activity against MAb F13P13F was observed in lanes 4,6,7 and 8, lanes 3 and 5 showed no activity with very little background. This is of some concern as lane 3 contained the strain from which the MAb was derived and lane 5 contained an FD2 lyophilised isolate which appeared identical in the protein gels. The above experiment was repeated with modified sample loadings.

SDS-Page and Western Blot Analysis of Isolates and LPS Fragment

Same samples and conditions as before, however, 1.5 mm gels were used in order to achieve a higher sample loading. This is a repeat of the previous experiment.

Gel 1 using unstained standards for Coomassie Blue (Coom.)
Gel 2 using prestained standards for Western blot transfer. (W/B)

Lane	Sample	Vol (µL)	
		W/B	Coom.
1	LMW Stds	5	5
2	LMW Stds	5	5
3	VAN01 ex Deakin	30	20
4	*V. anguillarum* BAC_NSF 317	25	15
5	*V. anguillarum* Fish Vibrio 7/80	35	20
6	*V. anguillarum* Fish Vib	40	20
7	*V. anguillarum* BAC_NSF 32	35	20
8	VAN01 LPS frag. (prepared 25/5/93)	35	0 (5.6µg LPS)
9	LMW Stds	5	5
10			

Western blot: (standard conditions, m:\met\page\wblot NG)
Transferred at 4°C O/N at 10 volts.
TCSN MAb F13P13F, undiluted 2 hours at 37°C.
Silenus S anti Mouse,HRPO @ 1:500

Electrophoresis result

Result was consistant with the previous results. Samples appeared as expected. Samples were reasonably well balanced in the relative amounts loaded.

Western blot result

Single well developed bands at approximately 48kDa in lanes 3,4,5 and 7. Lanes 6 and 8 showed no activity. Lane 8 contained LPS fragment. No other background or bands.

Isolate Mt. Pleasant 85/3954-1 was reisolated from rainbow trout inoculated with bacteria twice passaged through fish.

Bacteria was cultured on SBA at 22°C in air, harvested into sterile PBSA and washed 3 times in PBSA, prior to solubilization in SDS-PAGE sample buffer. Sample labelled *V. anguillarum*, ex Rainbow trout H1.

SDS-PAGE AND WESTERN BLOT ANALYSIS

Experiment Comparison of *V. anguillarum* whole cell preps with challenge isolate, isolate from challenged fish and VAN01 LPS preps.

Electrophoresis Laemlli,U.K. (1970) Nature 227:680-685
2 X 12% (29:1) Acrylamide:PDA, 4.5cm X 0.75mm mini gel.
200V for 40 min. at ambient temperature.
SDS-PAGE Standards, low range Biorad 161-0304 LR

Sample Preparation

Cells from SBA cultures ex challenge inoculum culture (72h) and SBA culture ex infected rainbow trout (48h) were washed 3 times in PBSA, resuspended in 200µL of PBSA and heated with an equal volume of 2X sample buffer at 100°C for 5 minutes. VC=*V. anguillarum* challenge isolate, VI=*V. anguillarum* isolated from challenged fish.

Gel 1 - Transfer

Lane	Sample	Volume(µL)
1	PSLR	5
2	VC	10
3	VI	10
4	VAN01 whole LPS	15
5	LPS frag	20
6	PSLR	5
7	VC	10
8	VI	10
9	VAN01 whole LPS	15
10	LPS frag 25/5/93 (NG)	20

Gel 2 - LPS stain

1	LR	5
2	VC	10
3	VI	10
4	VAN01 whole LPS	15
5	LPS frag	20

Gel Stain LPS Stain

Results

LPS was detected in all test lanes, including the whole cell lysates from VC and VI. The majority of LPS was detected in the purified whole LPS sample. The LPS fragment did not stain as excepted. No LPS band was observed at around 14-17 kDa but 2 faint but distinct bands were observed at around 67 kDa, these bands were associated with smearing typical of LPS and corresponded to LPS bands observed in the whole LPS preparation. The amount of LPS appeared to be greatest in the whole LPS preps. while the cell lysate demonstrated significant amounts of LPS. The sample from purified LPS fragment did not stain intensly and it appeared that there was significantly less LPS in this sample. This would explain the apparent failure of the MAb to detect the purified fragment (MAb F14 did detect LPS in the cell lysate and the whole LPS prep, but not in the purified LPS fragment sample.)

Conclusions

There appeared to be 1/50-1/100 less LPS in the purified fragment sample than in either the whole LPS prep. or the cell lysates.

It appears that the purified LPS fragment underwent a degree of reassociation as no band was observed below 20 kDa but there was some material at higher MW's.

Western Blot

Buffer System Towbin *et al.* (1979) PNAS 76:4350

Transfer Conditions 30 volts for 3 hours at approximately $4^{O}C$.

Reagents

Blocking solution/Ab diluent, sedimented 3% skim milk, Tris/saline, pH 7.4 (Ab SM)
Blot 1 - 1^{O} antibody, hybridoma TCSN F13P13F (previously unopened)
Blot 2 - 1^{O} antibody, hybridoma TCSN F14P55D
2^{O} antibody conjugate, anti-Mouse HRP (Silenus), @ 1:1000 in Ab SM
Wash buffer 1, Tris-saline pH 7.4
Wash buffer 2, Tris-saline, 0.05% v/v tween 20, pH 7.4
Substrate, 4-chloro-1-napthol solution.

Procedure

1. Block with Ab Skim Milk overnight at $4^{O}C$.
2. React with 1^{O} antibody, 60 minutes at $37^{O}C$.
3. Wash with Tris/saline/tween, 2 X 3 minutes.
4. Wash with Tris/saline, 1 X 3 minutes.
5. React with 2^{O} antibody HRP conjugate, 60 minutes at R/T.
6. Wash with Tris/saline/tween, 2 X 5 minutes.
7. Wash with Tris/saline, 1 X 5 minutes.
8. Develop with 4-chloro-1-napthol.
9. Stop and wash with distilled water, 5 min. and dry on filter paper.

Results

The blot with MAb F14 displayed three faint bands at approx. 14 kDA corresponding to the LPS in VC, VI and the whole LPS prep., no activity was observed in the purified LPS fragment lane. Previous blots using whole cell lysates and the purified LPS fragment with MAb's F13 and F14 revealed several regions of activity. At approx. 50 kDA, 32 kDa, 20kDa and one at around 18 kDA (a weak band corresponding to the fragment)

Conclusions

The apparent lack of activity in the blots was attributed in part to the concentration of antigen bound to the membrane. The stained gels revealed a significant difference in the amount of LPS present in the samples and this would account for the absence of any reaction in the purified LPS fragment sample. The stained gels also revealed that the LPS fragment was not present in a low molecular weight form and hence no activity against the MAb's was observed at around 14 kDa.

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Development of Monoclonal Antibodies That Identify *Vibrio* Species Commonly Isolated from Infections of Humans, Fish, and Shellfish

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Monoclonal antibodies (MAbs) against *Vibrio* species that infect humans, fish, and shellfish were developed for application in rapid identifications. The pathogens included *Vibrio alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. cholerae*, *V. damsela*, *V. furnissii*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, and *V. vulnificus*. Three types of MAbs were selected. The first important group included MAbs that reacted with only a single species. A second group comprised a number of MAbs that reacted with two, taxonomically closely related *Vibrio* species. For example, of 22 MAbs raised against *V. alginolyticus*, 6 recognized a 52-kDa flagellar H antigen common to both *V. alginolyticus* and *V. parahaemolyticus*; *V. anguillarum* and *V. ordalii* also shared antigens. A third group included three genus-specific MAbs that reacted with almost all *Vibrio* species but did not react with other members of the family *Vibrionaceae* (e.g., members of the *Aeromonas*, *Photobacterium*, and *Plesiomonas* genera) or a wide range of gram-negative bacteria representing many genera. This last group indicated the possible existence of an antigenic determinant common to *Vibrio* species. Two of these three genus-specific MAbs reacted with heat-stable antigenic determinants of *Vibrio* species as well as lipopolysaccharide extracted from *Vibrio* species. The use of the MAbs in blind tests and diagnosis of clinical isolates indicated that three different types of bacteria, viz., live, formalin-fixed, and sodium azide-killed bacteria, were detected consistently. Overall, it was found that the genus-specific MAbs were very useful for rapidly identifying vibrios in the screening of acute infections, while the species-specific MAbs and others were useful for completing the diagnosis.

Although *Vibrio* species are predominantly found in aquatic environments, they can also be isolated from infected humans and marine animals. While many of the species are thought to be harmless to humans, some are considered to be potentially dangerous. Ten *Vibrio* species have been linked to human infections, including outbreaks of severe gastroenteritis, localized wound infections, and systemic infections resulting in primary and secondary bacteremia and septicemia. Species associated with the human infections are *Vibrio alginolyticus*, *V. cholerae* O1 and non-O1, *V. damsela*, *V. fluvialis*, *V. furnissii*, *V. hollisae*, *V. metschnikovii*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus* (29). The species that have been associated with fish and shellfish diseases include *V. alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. cholerae* non-O1, *V. damsela*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, and *V. salmonicida* (3, 11, 13, 24).

The genus *Vibrio* is represented by 29 species (23, 25). Numerical classification schemes for the family *Vibrionaceae*, which includes the four genera *Vibrio*, *Aeromonas*, *Photobacterium*, and *Plesiomonas*, have been proposed by Baumann et al. (4) and Bryant et al. (6). An identification scheme (7) based on a matrix of 81 characters resulted in the identification of 38 phenons within the family *Vibrionaceae*, of which 29 correlated with previously defined *Vibrio* species.

The normal identification of pathogenic vibrios in the environment has involved a four-step strategy: (i) collection of the samples, (ii) recovery of vibrios from the samples, (iii) identification of the vibrios recovered, and (iv) confirmation that they are pathogenic (36). The preliminary differentiation of *Vibrio*-like organisms from other gram-negative bacteria has involved the growth of isolates on thiosulfate-citrate-bile salts (TCBS) agar selective medium, on which most vibrios will grow. Further differentiating characteristics include salt requirement for growth, gelatinase production, sensitivity to the vibriostatic compound 0/129 (2,4-diamino-6,7-diisopropylpteridine phosphate), and an oxidase-positive reaction. Confirmatory tests often utilize serological reactions, such as agglutination tests (36). Identification of *Vibrio* species that possess similar morphological, physiological, and biochemical characteristics still remains a problem because of the large number of tests that are involved and that usually give an identification with a probability level of less than 100%. Alternative serological identifications of *Vibrio* species with polyclonal antisera have resulted in relatively quick results (2), but there are often limitations due to nonspecificity (19).

Rapid identification of pathogenic strains facilitates better management of infection and understanding of disease etiology. In order to meet these demands, monoclonal antibodies (MAbs) were developed against *V. alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. cholerae*, *V. damsela*, *V. furnissii*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, and *V. vulnificus*, to be used in enzyme-linked immunosorbent assay (ELISA) and fluorescein isothiocyanate (FITC) immunofluorescence tests.

MATERIALS AND METHODS

Bacterial sources. A wide range of *Vibrio* strains were obtained from the Australian Collection of Marine Microorganisms at the Sir George Fisher Centre for Tropical Marine Studies, James Cook University of North Queensland, Townsville, Australia; the Australian Fish Health Reference Laboratory at the Australian Animal Health Lab-

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oratory, Geelong, Australia; the Department of Microbiology, University of Queensland, Brisbane, Australia; the Department of Microbiology, University of Adelaide, Adelaide, Australia; the Fish Health Unit, Department of Primary Industry, Launceston, Tasmania, Australia; the Department of Clinical Bacteriology, Huddinge University Hospital; the Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark; and the Royal North Shore Hospital, Sydney, Australia. Additional gram-negative bacteria were obtained from the Geelong Hospital, Geelong, Australia.

Media for the maintenance of bacterial cultures. Cultivation of large volumes of vibrios was carried out with luminous medium developed from basal medium (30). It contained 500 ml of filtered seawater, 50 ml of 1 M Tris buffer at pH 7.5, 0.025 g of NH_4Fe citrate, 0.075 g of $K_2HPO_4 \cdot 3H_2O$, 1 g of NH_4Cl, 3 ml of glycerol, 5 g of tryptone, 5 g of yeast extract (Oxoid), and 450 ml of distilled water.

Vibrio isolates were maintained on luminous agar, i.e., luminous medium with 1.2% (wt/vol) agar, and subcultured bimonthly. At each subculturing, bacterial strains were tested for the ability to grow on selective TCBS agar (Oxoid). Long-term storage of strains was achieved by adding sterile glycerol, to a final concentration of 30% (vol/vol), to log-phase cultures and then freezing each in 2-ml ampoules at −80°C. Other gram-negative bacteria were cultivated on tryptone soya agar (Oxoid) and tryptone soya broth (Oxoid) media.

Preparation of bacteria for immunization and screening. Overnight cultures grown in luminous media were centrifuged at 10,000 × *g* in a Beckman J2-21M/E centrifuge at 4°C for 20 min. The supernatant was discarded and the bacteria were washed three times with phosphate-buffered saline (PBS) at pH 7.3, each time being centrifuged at 10,000 × *g* for 20 min. The final pellet was resuspended in 50 ml of PBS. The bacteria were then killed by the addition of 0.1% (wt/vol) sodium azide and stored at 4°C until used.

MAb production. Immunization protocols in which 10^8 sodium azide-killed and PBS-washed vibrios were suspended (1:1) in Freund's complete adjuvant and then injected intraperitoneally into 8-week-old female BALB/c mice once a week for 3 weeks were employed (14). On day 21, viz., 4 days before the hybridization, the mice were given a booster injection of 10^8 *Vibrio* bacteria resuspended in PBS without adjuvant. Mice were bled from the retro-orbital sinus prior to a fusion to test antibody titers in sera by indirect ELISA. Fusions were performed when antiserum titers were 1:1,000 or higher.

The hybridization protocols of De St. Groth and Scheidegger (12) and Gustafsson et al. (15) were followed, with a few modifications. The spleens of immunized mice were removed under sterile conditions, placed in RPMI 1640 medium (Flow) containing penicillin at 100 IU/ml and streptomycin at 100 μg/ml, cut into pieces, and then sieved through a sterile metal mesh to obtain a fine suspension. The cells were washed in RPMI 1640 medium and counted in a cell counting chamber (Neubauer). Cells of the non-immunoglobulin (Ig)-secreting murine hypoxanthine guanosine phosphoribosyl transferase-deficient myeloma cell line Sp2/O-Ag14 (32) were washed in the same medium prior to mixing 0.5 × 10^7 cells with 10^7 spleen cells. The cell mixture was centrifuged at 150 × *g* for 10 min, and the supernatant was discarded. A 1-ml filter-sterilized sample of 45% (wt/vol) polyethylene glycol 4000 (Merck 9727) was prepared on the day of the fusion by dissolving polyethylene glycol 4000 in RPMI 1640 medium. It was slowly added to the cell mixture, with gentle shaking during 60 s, and after a further 90 s at 37°C the polyethylene glycol 4000 was diluted by the slow addition of RPMI 1640 medium. The cells were then pelleted by centrifugation at 100 × *g* for 10 min; the supernatant was discarded; the cells were resuspended in 50 ml of prewarmed RPMI 1640 medium containing hypoxanthine, aminopterin, and thymidine and 10% (vol/vol) fetal calf serum (Flow); and then the cells were dispensed in 100-μl aliquots into 96-well microtiter plates. They were incubated in a tissue culture incubator at 37°C with 80% humidity, 5% CO_2, and 95% air. Murine peritoneal macrophages were employed as feeder cells to facilitate hybridoma growth and survival.

Cloning. First clonings of hybridomas were carried out at a cell density of 20 cells per ml in hypoxanthine and thymidine medium by the limiting dilution method (14). Positive subclones were selected for a second round of cloning by a modified procedure of the first cloning. The initial cell density of hybridoma cells was reduced to 10 cells per ml in RPMI 1640 medium, resulting in approximately 0.45 to 0.55 cells per well.

Production of ascites. Six- to 8-week-old male BALB/c mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma) by the method of Hoogenraad and Langdon (18). The mice were then allowed to rest for 7 days before being injected intraperitoneally with 10^7 hybridoma cells. Production of ascites was monitored daily from 4 days after the injection of the hybridoma cells. Ascitic fluid was collected once daily with an 18-gauge needle, centrifuged at 1,000 × *g* for 10 min, and stored in the presence of 0.02% (wt/vol) sodium azide at 4°C.

Homologous ELISA for screening hybridomas. At approximately 12 days posthybridization, supernatants were screened against homologous bacterial strains (20). ELISA plates were coated in a cold room overnight with the homologous bacterium, viz., 100 μl of washed bacterial cells (A_{620} = 0.2), suspended in coating buffer, per well. The wells were blocked by the addition of 100 μl of 1% (wt/vol) bovine serum albumin (Sigma) at room temperature (RT) for 4 h. Immediately prior to use, the plates were washed three times with a washing solution of 0.9% NaCl–0.05% Tween 20. Hybridoma supernatants were then added at 100 μl per well to the ELISA plates and incubated at RT for 4 h. This was followed by three washes with washing solution and the addition of 100 μl of goat anti-mouse Ig-alkaline phosphatase conjugate (Sigma), diluted 1:1,000 in incubation buffer, per well. After the plates had been incubated at RT overnight, unreacted enzyme conjugate was removed from the wells by three washes with washing solution. Finally, 100 μl of substrate solution containing *p*-nitrophenyl-phosphate substrate (Sigma) was added to each well and the plates were incubated at 37°C for 100 min. The optical densities were then read at 405 nm on a Flow MCC 340 plate reader, and MAbs were considered to be positive when results were 0.6 or greater after subtracting controls. Corresponding clones were transferred to 24-well plates to multiply.

Heterologous ELISA for determining MAb specificity. The supernatants of 24-well plates were then rescreened in a heterologous ELISA against a wide range of *Vibrio*, *Aeromonas*, *Photobacterium*, and *Plesiomonas* species and other gram-negative bacteria to determine clones that showed potential use in *Vibrio* identification. The protocols were the same as those of the homologous ELISA.

Rapid identification of vibrios. Live and formalin-fixed vibrios were used in direct and indirect ELISA as used for screening and were also used in indirect FITC immunofluorescence testing. However, each incubation step was carried

TABLE 1. *Vibrio* species for which MAbs have been developed[a]

Vibrio species	Strain	Source
V. alginolyticus	ACMM 101	JCUNQ
V. anguillarum	AFHRL 1	AAHL
	AFHRL 43	AAHL
V. carchariae	ATCC 35084	JCUNQ
V. cholerae O1	Inaba 569B	HUH
V. damsela	ATCC 33537	JCUNQ
V. furnissii	ATCC 35016	JCUNQ
V. harveyi	ACMM 130	JCUNQ
V. ordalii	ATCC 33509	JCUNQ
V. parahaemolyticus	WPI	HUH
V. vulnificus	ACMM 106	JCUNQ

[a] Abbreviations: ACMM, Australian Collection of Marine Micro-organisms; AFHRL, Australian Fish Health Reference Laboratory; ATCC, American Type Culture Collection; JCUNQ, James Cook University of North Queensland; AAHL, Australian Animal Health Laboratory; HUH, Huddinge University Hospital.

out at RT, and the time was reduced to 30 min for ELISA or less for FITC tests, thus producing final results within 3 h.

Isotyping of MAbs. Each clone was grown in Monomed serum-free medium (CSL) to provide a source of semipure Ig for isotyping. The isotype of each MAb was determined by use of an isotyping kit (Bio-Rad) and confirmed by Ouchterlony double diffusion with anti-mouse sera (Sigma). Ouchterlony gel double diffusion was performed on slides by using 0.85% (wt/vol) agarose type vii (Sigma) in 50 mM PBS at pH 7.4 and containing 0.02% (wt/vol) sodium azide.

Determination of surface antigens recognized by MAbs. In order to gain information regarding the nature of *Vibrio* surface antigens recognized by specific MAbs, sodium azide-killed *Vibrio* suspensions were heated at 100°C for 2 h. This denatured heat-labile antigenic components such as proteins, leaving heat-stable antigens such as lipopolysaccharide (LPS) on the surface. Both heat-treated and unheated bacteria were tested with MAbs in a conventional ELISA. If the final optical density of the heat-treated bacteria was greater than 50% of that of the unheated bacteria, the antigen was considered to be heat stable. Conversely, it was defined as being heat labile when the optical density was less than 25% of that of the unheated bacteria, while an optical density between these values were thought to represent a partially heat-stable antigen.

Flagellum and LPS preparations. *Vibrio* flagella were prepared by the method of Yancey et al. (44). LPS was extracted and purified by the method described by Westphal et al. (43).

Electrophoresis and immunoblots of flagella and LPS. Samples of LPS or flagella were diluted in sample buffer, and 10 μg per lane was separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis for 1 h at 200 V (21). Separated components were visualized by silver staining (17). Additional gels were run, and the separated components were transferred to nitrocellulose sheets for immunoblot analyses (41). Prestained low-molecular-weight standards (Bio-Rad) were used to monitor separations and transfers and to assess molecular weights.

RESULTS

Production of MAbs. MAbs were raised against 10 pathogenic *Vibrio* species (Table 1). Each fusion produced at least two, and in several cases many, positive hybridomas. Altogether, 102 MAbs were selected for potential diagnostic purposes after the MAbs that reacted with several species had been discarded. The distributions of Ig heavy chains of the selected MAbs were 49 (48.0%) IgG1, 12 (11.8%) IgG2a, 10 (9.8%) IgG2b, 14 (13.7%) IgG3, 13 (12.7%) IgM, 2 (2.0%) IgA, and 2 (2.0%) showing both IgM and IgG2b. The numbers of κ and λ light chains were 99 (97.0%) and 3 (3.0%), respectively.

Of the 102 MAbs selected, 55 (53.9%) recognized heat-stable antigens, 37 (36.3%) reacted with heat-labile antigens, and 10 (9.8%) were found to be partially heat stable. MAbs that recognized heat-stable epitopes of *Vibrio* species also reacted in ELISA with LPS extracted from the same species, including *V. alginolyticus*, *V. anguillarum*, *V. cholerae* O1, *V. furnissii*, *V. ordalii*, and *V. parahaemolyticus*.

The 102 MAbs of use in *Vibrio* identifications were divided into three groups after cross-screening against gram-negative bacteria (Table 2). Each showed a definitive pattern of immunoreaction. The first important group showed species and often strain specificities. Another group recognized only one other closely related species, whereas the third group of MAbs cross-reacted with nearly all bacteria within the genus *Vibrio*. An additional MAb that recognized nearly all gram-negative bacteria was also retained for future research.

Species- and strain-specific MAbs. Species-specific MAbs recognized only the bacterial species originally used to immunize and produce the MAbs. We now have MAbs that are useful in identifying *V. alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. cholerae* O1, *V. damsela*, *V. furnissii*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, and *V. vulnificus* (Table 1).

However, where there were many strains within a species, some species-specific MAbs were found to react with all strains, whereas others reacted with only some strains (Tables 3 and 4). For example, a fusion following immunization with the *V. cholerae* O1 Inaba 569B strain produced a species-specific MAb, F7P511C, that reacted with all O1 and non-O1 strains; another MAb, F7P25A, that reacted with O1 but no non-O1 strains; and a third MAb, F7P510D, that reacted with O1 Inaba strains only (Table 3). Immunoblotting of *V. cholerae* cell lysates confirmed the ELISA results, and the identification of *V. cholerae* O1 strains is shown in Fig. 1.

In another example, we have obtained 14 species-specific MAbs from fusions in which two strains of *V. anguillarum*, AFHRL 1 and AFHRL 43, were used to immunize separate mice (Table 4). These MAbs recognized only the O1 serotype of 10 *V. anguillarum* serotypes obtained from Denmark (22) as well as the homologous strains used for the immunizations, which were themselves typed as O1. Of the four MAbs recognizing both immunizing strains, F13P13F and F13P77D reacted with all of our *V. anguillarum* isolates obtained from Australian and overseas sources and serotyped as O1 strains. These two MAbs are useful in *Vibrio* identification at the species level for *V. anguillarum*.

MAbs against antigens shared between two related *Vibrio* species. Some MAbs raised against a particular *Vibrio* species reacted with only one other closely related *Vibrio* species. For example, 22 MAbs reacting with *V. alginolyticus* also reacted with *V. parahaemolyticus*. Several of these MAbs reacted in immunoblots with an antigen of 52 kDa extracted from the flagella of both species (Fig. 2). These data are consistent with a flagellum H antigen being shared by these two *Vibrio* species (4). The MAbs reacting with the H antigen have been useful in identifying new isolates. The species *V. alginolyticus* and *V. parahaemolyticus* were

TABLE 2. Gram-negative bacterial strains used in cross-screening of MAbs

Genus	Species and strain(s) (source)[a]
Aeromonas	*A. caviae* 2782 (PMCMRF)
	A. hydrophila AFHRL 85:584-1A, AFHRL 85:8438 (AAHL)
	A. salmonicida AFHRL 1107-1B, AFHRL 85:9370-A, AFHRL 86:674-3339 (AAHL); 166 (PMCMRF)
	A. sobria AFHRL 86:2208-18, AFHRL 86:2240-A (AAHL); 2397 (PMCMRF)
Enterobacter	*E. cloacae* 907 (GH)
Escherichia	*E. coli* 900 *lac* mutant (GH); 907 *lac*$^+$ (GH)
Klebsiella	*K. pneumoniae* 744 (GH)
Photobacterium	*P. angustum* ACMM 63 (JCUNQ)
	P. leiognathi ACMM 51 (JCUNQ)
	P. phosphoreum ACMM 50 (JCUNQ)
Plesiomonas	*P. shigelloides* 3973 (GH)
Proteus	*Proteus* sp. strain 89-3553 (GH)
Pseudomonas	*P. aeruginosa* 648 (GH)
	Pseudomonas sp. strain 11380 (GH)
Salmonella	*S. paratyphi* group A 8570, group B 14733, group C 16686 (GH)
	S. typhi 17239 (GH)
Shigella	*S. dysenteriae* 3-10 (GH)
	S. flexneri 6561 (GH)
	S. sonnei 31356 (GH)
Vibrio	*V. alginolyticus* AMC/85 (FHU); ACMM 101 (JCUNQ); 12331 (RNSH)
	V. anguillarum AFHRL 1, AFHRL 43, AFHRL 44, AFHRL 45, AFHRL 46, AFHRL 47 (AAHL); 86.2761 (FHU); O1 ATCC 43305, O2 ATCC 43306, O3 ATCC 43308, O4 ATCC 43308, O5 ATCC 43309, O6 ATCC 43310, O7 ATCC 43311, O8 ATCC 43312, O9 ATCC 43313, O10 ATCC 43314 (RVAU); ATCC 2628, ATCC 19268, 2743, 2744, 2843, 3247, 3248, 3250, 3251, 3260 (UQ)
	V. carchariae ATCC 35084 (JCUNQ)
	V. cholerae Inaba 569B, Brazil 1198-78, El Tor Ogawa 3083, non-O1 V3 (HUH)
	V. damsela 89.773 (FHU); ATCC 33537 (JCUNQ)
	V. fischeri ACMM 136 (JCUNQ)
	V. fluvialis NCTC 11327 (JCUNQ); 11295 (RNSH)
	V. furnissii ATCC 35016 (JCUNQ)
	V. harveyi 7.919 (FHU); ACMM 130 (JCUNQ)
	V. hollisae ATCC 33565 (JCUNQ)
	V. mediterranei 87.919 (FHU)
	V. nereis 88.4523 (FHU)
	V. ordalii ATCC 33509 (JCUNQ)
	V. parahaemolyticus WPI S-14186, FC 1011, 3D38 (HUH); 9472 (RNSH)
	V. splendidus 88.3837 (FHU); ACMM 140 Bio. I (JCUNQ)
	V. vulnificus (medical isolate) (HUH); ACMM 106 (JCUNQ); ATCC 33148, 11308, 11309 (RNSH); 2740, 2741, 2743, 2745, 3060, 3061 (UQ)

[a] Sources are in parentheses. Abbreviations: PMCMRF, Princess Margaret Children's Medical Research Foundation; GH, Geelong Hospital; FHU, Fish Health Unit, Department of Primary Industry; UQ, University of Queensland; RVAU, Royal Veterinary and Agricultural University; RNSH, Royal North Shore Hospital. For other abbreviations, see Table 1, footnote *a*.

originally classified as *V. parahaemolyticus* biotypes 1 and 2 but have now been reclassified as two species (34).

In another example, MAbs F18P66C and F18P82G raised against *V. ordalii* were found to react with *V. ordalii* ATCC 33509 and *V. anguillarum* serotype O2 strains (Table 4). This was confirmed in immunoblots with cell lysates of *V. ordalii* ATCC 33509 and *V. anguillarum* O1 and O2 strains, of which only *V. ordalii* and *V. anguillarum* O2 were positive (Fig. 3). Together, these data are consistent with previous findings (9) that the two species share a surface antigen. The current research showed that only O2 strains of *V. anguillarum* possessed a shared antigen with *V. ordalii*.

TABLE 3. Specificities of MAbs recognizing strains of *V. cholerae*

MAb	Result with:					MAb specificity
	O1 strain				Non-O1 strain	
	Inaba 569B	Brazil	Ogawa	Rough mutants[a]		
F7P511C[b]	+	+	+	+	+	Species
F7P25A	+	+	+	+	−	All O1 strains
F7P510D	+	−	−	+	−	Inaba strains
F11P411F[c]	+	+	+	+	+	Genus

[a] Inaba 569B rough strains provided by P. Manning, University of Adelaide.

[b] MAb does not react with other *Vibrio* species.

[c] MAb reacts with all *Vibrio* strains except *V. damsela*.

Genus-specific MAbs. Genus-specific MAbs recognized *Vibrio* species but no other species within the family *Vibrionaceae* (i.e., *Aeromonas*, *Photobacterium*, and *Plesiomonas* species) or other gram-negative bacteria. Two of these MAbs, F11P411F [IgG1(κ)] and F7P52C [IgG2a(κ)], reacted strongly with *Vibrio* strains listed in Tables 1 to 4 and many new *Vibrio* isolates, except for *V. damsela* strains. They recognized live, sodium azide-killed, or formalin-fixed *Vibrio* species in ELISA and FITC immunofluorescence microscopy. They were also found to recognize heat-stable epitopes on the surfaces of the bacteria. Furthermore, they reacted in ELISA and immunoblots with LPS extracted from six *Vibrio* species.

Another MAb, F7P63B [IgM(κ)], reacted only with heat-labile antigenic determinants of *Vibrio* species. All three of

TABLE 4. ELISA cross-screenings of *V. anguillarum* serotypes O1 to O10 with MAbs produced against *V. anguillarum* and *V. ordalii*

MAb	Result with:												
	V. anguillarum serotype										*V. anguillarum*		*V. ordalii* ATCC 33509
	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	AFHRL 1	AFHRL 43	
Produced against *V. anguillarum* AFHRL 1													
F13P18A	++	−	−	−	−	−	−	−	−	−	+++	−	−
F13P13F	+++	−	−	−	−	−	−	−	−	−	+++	+	−
F13P212C	+++	−	−	−	−	−	−	−	−	−	+++	−	−
F13P49H	++	−	−	−	−	−	−	−	−	−	+++	−	−
F13P410C	+	−	−	−	−	−	−	−	−	−	+++	−	−
F13P411C	+	−	−	−	−	−	−	−	−	−	+++	−	−
F13P56D	++	−	−	−	−	−	−	−	−	−	+++	−	−
F13P58A	++	−	−	−	−	−	−	−	−	−	+++	−	−
F13P610F	+	−	−	−	−	−	−	−	−	−	++	+	−
F13P611F	++	−	−	−	−	−	−	−	−	−	+++	−	−
F13P77E	+++	−	−	−	−	−	−	−	−	−	+++	−	−
Produced against *V. anguillarum* AFHRL 43													
F14P110B	+	−	−	−	−	−	−	−	−	−	−	+++	−
F14P55D	+++	−	−	−	−	−	−	−	−	−	+++	+++	−
F14P512D	−	−	−	−	−	−	−	−	−	−	−	++	−
Produced against *V. ordalii* ATCC 33509													
F18P66C	−	+++	−	−	−	−	−	−	−	−	−	−	+++
F18P82G	−	+	−	−	−	−	−	−	−	−	−	−	+

the MAbs have been useful in rapid identification of *Vibrio* species in new isolates and those held in stock.

MAb cross-reacting with all gram-negative bacteria. MAb F6P13G [IgM(κ)] was originally raised against *V. parahaemolyticus*. It reacted with more than 80 bacterial strains, including the four genera of the family *Vibrionaceae* and a range of enteric pathogenic gram-negative bacteria. Only six bacteria were not recognized by this MAb, of which two were *Vibrio* and four were *Aeromonas* species. The significance of the broad cross-reactivity is unknown. There are reports of antibodies reacting against lipid A, which is common to all gram-negative bacteria (38), but the true nature of the cross-reacting common antigen in this study is still undefined.

DISCUSSION

Identifications of *Vibrio* bacteria have been described by a number of research groups in recent years (7, 23, 25, 42). These identifications have played important roles but have usually incorporated many techniques and required adequate microbiological and immunological skills of the laboratory staff. However, during the 1980s, MAbs were produced against a variety of bacteria and used for rapid identification of the bacteria. For example, some MAbs have been developed against *V. anguillarum* and *V. salmonicida*

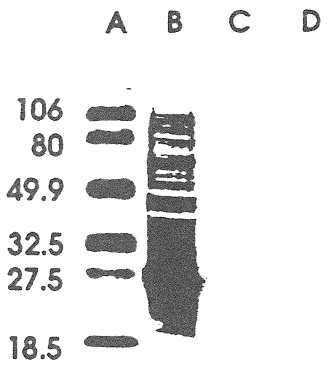

FIG. 1. Immunoblot of lysates of *V. cholerae* O1 and non-O1 strains with MAb F7P25A produced against *V. cholerae* O1 (Inaba 569B). The MAb reacted with the O1 strain only. Lanes: A, prestained standards (Bio-Rad), with sizes (in kilodaltons) indicated on the left; B and C, *V. cholerae* O1 and non-O1, respectively; D, *V. anguillarum* serotype O1 as a control.

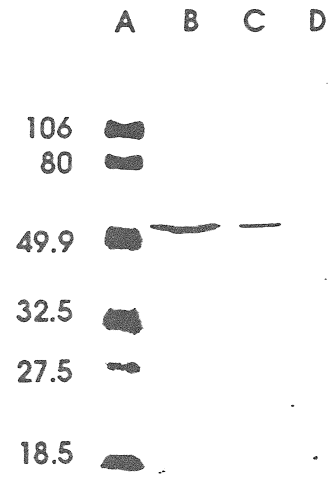

FIG. 2. Immunoblot of flagellum preparations of *V. parahaemolyticus* and *V. alginolyticus* with a MAb, F15P710G, produced against whole cells of *V. alginolyticus*, showing that the MAb reacts with a 52-kDa H antigen. Lanes: A, prestained standards (Bio-Rad), with sizes (in kilodaltons) indicated on the left; B and C, *V. parahaemolyticus* FC1011 and *V. alginolyticus* ACMM 101 flagella, respectively; D, culture medium.

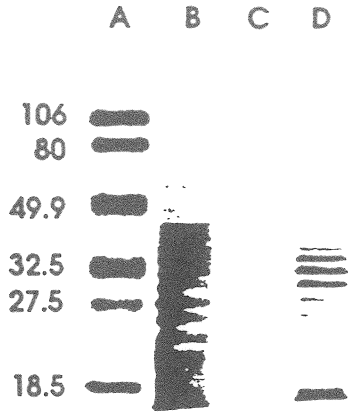

FIG. 3. Immunoblot of lysates of *V. ordalii* ATCC 33509, *V. anguillarum* serotype O1, and *V. anguillarum* serotype O2 with a MAb, F18P66C, produced against *V. ordalii* ATCC 33509. *V. ordalii* and *V. anguillarum* serotype O2 share antigens. Lanes: A, prestained standards (Bio-Rad), with sizes (in kilodaltons) indicated on the left; B through D, *V. ordalii* ATCC 33509, *V. anguillarum* serotype O1, and *V. anguillarum* serotype O2, respectively.

(5, 13, 39), but most have been developed against *V. cholerae* O1 (1, 15, 19, 27). Success in the identification of some *Vibrio* species has occurred by use of H antisera and antiflagellar MAbs (28, 33, 40).

We have developed MAbs against 10 pathogenic *Vibrio* species for use in rapid identifications of the bacteria. More than 80% of these were of an IgG subclass and nearly all possessed κ light chains, which is consistent with previous studies of light chain production in mice (8). Of the 102 MAbs selected from those produced, about half recognized heat-stable epitopes, indicating that these surface antigens were LPS rather than protein. MAbs reacting with heat-labile epitopes were considered to recognize proteins, and this was supported by the recognition of *Vibrio* flagellar antigens analyzed in immunoblotting.

MAbs that were species specific and genus specific have been particularly useful in the identification of vibrios. On arrival in our laboratory, all unknown *Vibrio* isolates are first screened by MAbs specific to the genus and *Vibrio* positive strains are identified, whenever possible, by species-specific MAbs that have been produced against pathogenic vibrios. For example, in isolates supplied from four sources, the genus-specific MAb F11P411F recognized the isolates as being *Vibrio* species, whereas the species-specific MAb F31P46F identified the isolates as *V. vulnificus*. In addition, the species-specific and genus-specific MAbs have been successfully applied in a blind test (16) and some have been used to study the attachment of vibrios to trout cells (10).

An important MAb produced was F7P511C, as it identified O1 strains of *V. cholerae* among all vibrios, including the non-O1 strains of *V. cholerae*. MAb F7P25A recognized all *V. cholerae* O1 strains. These MAbs are now being used in identification of *V. cholerae* O1 isolates in epidemiological studies.

The MAbs that cross-reacted with closely related *Vibrio* species indicated the existence of antigens common to the related species. Cross-reactivity with very closely related antigentic substances is well known. For example, *V. parahaemolyticus* and *V. alginolyticus* were found to share a 52-kDa antigen, known as the H antigen (4). In the current research, 48% of the MAbs that were prepared against *V. alginolyticus* cross-reacted with *V. parahaemolyticus*. Many of these MAbs were found to react with a heat-labile surface antigen in flagellum preparations, indicating that the antigens were flagellum H antigens. Immunoblots confirmed that the MAbs recognized H antigen on flagella of both species. Similarities of the H antigen of other closely related *Vibrio* species have been shown in serological tests (37).

We found that two closely related species, *V. anguillarum* and *V. ordalii*, also shared antigens. In particular, MAbs produced against *V. ordalii* ATCC 33509 cross-reacted strongly with *V. anguillarum* serotype O2, but no serotype O1 strains, in both ELISA and immunoblotting. MAbs produced against *V. anguillarum* serotype O2 were found to cross-react with antigens on *V. anguillarum* O1 and *V. ordalii* (39). These two species were originally classified as *V. anguillarum* biotypes 1 and 2, respectively, but have now been reclassified as two species (31).

As mentioned in the Results, three MAbs recognized whole cells of nearly all *Vibrio* strains, implying that members of the genus *Vibrio* have a common epitope on their surfaces. One of these MAbs, F11P411F, reacted with live or dead cells (i.e., sodium azide treated, formalin fixed, and heat treated) as well as LPS extracted from a number of *Vibrio* species. We have found this MAb to be extremely useful in the identification of *Vibrio* bacteria among isolates.

The nonrecognition of *V. damsela* strains by the genus-specific MAb F11P411F and others indicates the need for the reclassification of *V. damsela*. There has been evidence supporting its reassignment to the genus *Listonella* (26) and, more recently, the genus *Photobacterium* (35). Further immunological studies are being carried out in our laboratory to clarify the status of this species.

Cross-reactions of MAbs with members of the family *Vibrionaceae* were found. As some MAbs reacted with a few strains of *Aeromonas* and *Photobacterium*, as well as most *Vibrio* strains, it is possible that an epitope is shared by these genera. With the exception of MAb F6P13G, no MAbs produced against vibrios cross-reacted with *Plesiomonas*. Finally, one MAb, F15P106D, cross-reacted with nearly all strains of *Vibrionaceae* as well as all other gram-negative bacteria tested. The significance of this is yet to be determined, but the MAb might recognize lipid, as gram-negative bacteria share a lipid A against which MAbs have been made (38).

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

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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 virulent strains of *A. hydrophila*. Antibodies from a clone, designated as F26P5C8, were found to identify the *A. hydrophila* serotype I isolates associated with EUS fish, and which were found to be virulent after subsequent inoculation studies. 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 is 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 freshwater 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* (Bloch), catfish, *Clarias batrachus* (L.), snakeskin gourami, *Trichogaster pectoralis* (Regan), crucian carp, *Carassius carassius* (L.), goby, *Glossogobius giurus* (Hamilton), sea mullet, *Mugil cephalus* L., flat-tailed mullet, *Liza dussumieri* (Valenciennes), bream, *Acanthopagrus australis* (Gunther), king threadfin, *Polydactylus sheridani* (Macleay), barramundi, *Lates calcarifer* (Bloch), rainbow trout, *Oncorhynchus mykiss* (Walbaum), trout cod, *Maccullochella macquariensis* (Cuvier & Valenciennes), and Macquarie perch, *Macquaria australasica* (Cuvier & Valenciennes) (Lio-Po *et al.* 1992; Llobrera & Gacutan 1987; Cahill 1987). In most cases, particularly in Australia, the virulence of *A. hydrophila* isolates was not determined and the role of *A. hydrophila* as a primary or secondary pathogen has not been established.

A number of virulence determinants have been identified and examined (reviewed by Cahill 1990). They include: the production of endotoxins such as lipopolysaccharides (LPSs), extracellular enterotoxins, haemolysins, cytotoxins, extracellular proteases, aerolysin, pili, adherence to cells and the possession of an S-layer surface protein. Inoculation studies with *A. hydrophila* from EUS have shown significant variation in the relative pathogenicity of isolates (Torres, Shariff & Law 1989). The importance of differentiating between pathogenic and

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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, Olivier & Lallier 1980). Subsequent studies indicate that virulent *A. hydrophila* strains possess a common O antigen (Lallier, Mittal, Leblanc & Lalonde 1981) associated with homogeneous length polysaccharide chains (Dooley, Lallier, Shaw & Trust 1985). Agglutination assays using heat-killed bacteria have indicated that virulent *A. hydrophila* isolates, associated with EUS, belong to a homogeneous serotype I group (Torres, Shariff & Tajima 1992). However, additional serogrouping of aeromonads has been unable to correlate virulence of the isolates with serogroups (Sakazaki & Shimada 1984; Misra, Shimada, Bhadra, Pal & Nair 1989). Given this phenotypic variation, there remains a clear need for the development of tests for markers of virulence for this group of organisms.

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

Materials and methods

Bacterial strains

Aeromonas hydrophila isolates used in this study to produce MAbs and carry out initial screenings (Table 1) were obtained from overseas sources in 2% (v/v) formalin to comply with quarantine regulations. Some additional isolates, including 85:584-1A and 85:8438, were supplied as live cultures from the Australian Fish Health Reference Laboratory (AFHRL), CSIRO, Geelong, Australia. A wide range of Gram-negative bacteria was 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 another 43 *A. hydrophila* isolates were screened at Hokkaido University.

The bacteria were grown aerobically, with agitation, at 37 °C in tryptone soya broth (Oxoid) to log phase, firstly in 10 ml volumes and subsequently in 1 l 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 13 000 rpm in an MSE microcentrifuge.

Immunizations

Two of the six virulent *A. hydrophila* isolates implicated with epizootic ulcerative disease (EUS) in South East Asia were strains 5 and 45 (Torres *et al.* 1989). Strain 45, isolated from the spleen of a diseased wild *Clarias* sp. from the Laguna de Bay, Philippines, was selected for the production of monoclonal antibodies.

The immunization protocol of Carlin & Lindberg (1983) was used. In the initial immunization, approximately 2×10^8 cells in 500 μl PBS were resuspended (1:1) together with Freund's complete adjuvant (CSL) and 500 μl 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 Freund's 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.

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.* (1992); U, unassigned serotype or untested; D, diseased fish; H, healthy fish; virulence was determined by injecting $6{\cdot}4 \times 10^4$ cells into healthy fish.

Media and culture conditions

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

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

Isolate[a]	Source	Indirect immunofluorescence[b]
85:584-1A	*Carassius auratus* (L.) (goldfish)	+
86:5879-G	*Lates calcarifer* Bloch (barramundi)	+
87:7281-6A	*Oncorhynchus mykiss* (Walbaum) (rainbow trout)	+
88:737-B	*Salmo trutta* L. (brown trout)	+
172	*Helostoma temmincki* Cuvier & Valenciennes (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)	+
84:12235-15F	*Salmo salar* L. (Atlantic salmon)	+ (weak)
84:12235-28F	*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.

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 & Köhler 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 0·45 μm 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). Additional ELISA assays were performed on a wide range of Gram-negative bacteria used in similar cross-screening tests (Chen, Hanna, Altmann, Smith, Moon & Hammond 1992).

Indirect immunofluorescent antibody staining

Five microlitres of bacterial strains, containing 10^8 cells ml^{-1} in PBS, were added to wells of

multiwell slides (Flow Laboratories). After air drying, cells were heat-fixed and 10 μl of test or isotype control hybridoma supernatants were added to the appropriate wells of the slides. Following incubation at room temperature (RT) for 30 min in a humidified chamber, slides were washed thoroughly with PBS and incubated at RT for 30 min with 20 μl of sheep anti-mouse $F(ab)_2$ conjugated with rhodamine (Silenus, DDR) diluted 1:50 in PBS containing 1% (w/v) BSA. The slides were then washed three times in PBS and mounted using 90% (v/v) glycerol and 4% (v/v) propyl gallate in PBS. Slides were examined for specific immunofluorescence using a Zeiss epifluorescence microscope.

ELISA screening

The specificity of hybridomas was examined by ELISA, as described by Carlin & Lindberg (1983). Briefly, bacterial isolates were washed three times in 0·05 M carbonate buffer, pH 9·6, adjusted to a final optical density of 0·4 at 620 nm (approximately 10^7 cells ml^{-1}), and 100 μl added to appropriate wells of a 96-well microtitre plate (Disposable Products, Adelaide, Australia). The bacteria were left to adsorb overnight at 4 °C before the plates were washed six times with 0·9% (w/v) NaCl containing 0·05% (v/v) Tween 20 (Selbys), and then blocked by the addition of 100 μl of PBS containing 1% (w/v) BSA (Sigma, A-7888) at 37 °C for 2 h. Plates were then washed six times as above, and 100 μl of hybridoma supernatant was added to the appropriate wells. Following incubation at RT for 1 h, plates were again washed six times, and 100 μl of goat anti-mouse immunoglobulin conjugated with alkaline phosphatase (Sigma, A-0162), diluted 1:1000 in PBS containing 1% (w/v) BSA, was added to each well and incubated at 37 °C for 1 h. Following a further six washes, bound antibody was detected by the addition of 100 μl of substrate solution containing 1 mg ml^{-1} *p*-nitrophenyl phosphate (Sigma) prepared according to the manufacturer's instructions. Following incubation at 37 °C for 100 min, absorbance was quantified at 405 nm using a Flow Multiscan MCC/340 microtitre plate reader. Results were considered positive if O.D.s were equal to, or greater than, 0·6 after subtraction of BSA block with substrate control values.

Characterization of the antigenic epitope

To determine the heat stability of the antigenic epitope recognized by F26P5C8 antibodies, washed preparations of sodium azide killed or formalin-fixed *A. hydrophila* in 0·5 M sodium carbonate buffer, pH 9·6, were boiled in a water bath for 2 h and then analyzed by indirect immunofluorescence and ELISA.

Lipopolysaccharide (LPS) was prepared from 1 l TBS cultures of the following *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 samples by centrifugation at 104 000 *g* for 3 h 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 280 nm. The LPS was then freeze-dried, made up to 1 mg ml^{-1} in sterile distilled H_2O, and stored at −70 °C until required.

Immunoreactivities of monoclonal antibodies to the LPS extracted from the two isolates was analysed by SDS-PAGE and immunoblotting of 10 μg samples. Additional samples of

blotted LPS were subjected to protein digestion using 0·25 mg ml^{-1} of proteinase K (Sigma) for 15 min at 37 °C prior to washing in TBS and subsequent immunoreactions being performed.

SDS-PAGE

SDS-PAGE was performed using a Mini-Protean II electrophoresis system (Bio-Rad) according to the method of Laemmli (1970). Initially, samples of *A. hydrophila* strains were washed three times in PBS by centrifugation at 13000 rpm for 5 min in an MSE microcentrifuge. The final pellets were then resuspended 1:1 in SDS-PAGE reducing sample buffer, vortexed vigorously, boiled for 3 min and centrifuged as above to remove any cell debris. Twenty microlitre samples corresponding to approximately 10^7 cells were separated electrophoretically using 12·5% reducing gels at a constant current of 10 mA per gel until the bromophenol blue dye front had reached the bottom of the gels. Resolved proteins were visualized with silver stain (Tsai & Frasch 1982). Molecular weights were estimated using SDS-PAGE standards (Bio-Rad, prestained low-range molecular weights).

Immunoblotting

Following SDS-PAGE, resolved components in unstained gels were transferred onto nitrocellulose sheets (Bio-Rad) at 30 V overnight (Towbin, Staehelin & Gordon 1979). Unbound reactive sites on the nitrocellulose sheets were blocked with 1% (w/v) BSA in TBS containing 10 mM Tris-HCl and 50 mM NaCl, pH 7·4, for 1 h. Sheets were then incubated for 1 h 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, DAH) diluted 1:2000 in TBS containing 1% (w/v) BSA, was then added and the sheets incubated at RT for 1 h. 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

Outbreaks of an EUS-like disease have been reported in Australia (Humphrey & Langdon 1986; Pearce 1987–1989), but the causative agent remains unknown. Although *A. hydrophila* has been associated with EUS outbreaks, it is generally considered to be an opportunistic pathogen. At present, little is known about the geographic distribution of the highly virulent strains of *A. hydrophila* recognized in South East Asia (Torres *et al.* 1989). In order to monitor the occurrence of disease caused by these strains and to develop effective quarantine strategies limiting the spread of disease, reliable diagnostic tests are required. The present study was initiated to develop a useful monoclonal antibody specific for pathogenic isolates of *A. hydrophila*.

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. In the initial screening, F26P5C8 also recognized two other *A. hydrophila* isolates, viz. EUS fish isolate 5 which was imported together with isolate 45, and

the Australian isolate 85:584-1A. The wide range of Gram-negative bacteria used in cross-screening ELISA tests were all negative.

Screening of Malaysian and Philippines 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). These isolates comprised most of the strains originally tested by Torres *et al.* (1989), of which 54 isolates, including six reference strains, were subjected to identification tests (Shotts & Bullock 1975; Popoff 1984) and subsequent virulence determination. Virulence was assessed by injecting juvenile grass carp, *Ctenopharyngodon idella* Valenciennes, of 10–20 g with 0·1 ml of $6{\cdot}4 \times 10^5$ cells of each isolate. Isolates that caused over 90% mortality within 7 days and in two trials were considered highly virulent. In the same trials, mortalities ranging from 50 to 89% during the test period were categorized as weakly virulent and mortalities less than 50% were considered avirulent.

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, reacted with MAb F26P5C8. Although these isolates originated from healthy fish, they were subsequently shown to be virulent in inoculation tests. Therefore, the current immunological tests correlated with 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. F26P5C8 also reacted with isolate 57, an *A. hydrophila*-like isolate which was virulent in inoculation tests. This isolate may be an atypical isolate of *A. hydrophila*, 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* L., ayu, *Plecoglossus altivelis* Temminck & Schlegel, 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 F26P5C8. 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. Therefore, it would be an important step to determine virulence and make comparisons with those from Malaysia and the Philippines.

In testing F26P5C8 with nine *A. hydrophila* isolates from cases of human diarrhoea, four isolates Ah-13, Ah-14, Ah-15 and Ah-16 were recognized. One isolate of *A. caviae* and 10 isolates of *A. sobria* from human diarrhoea were not recognized by F26P5C8. These data indicate the need for further studies to determine whether there is a possible aetiological link between *A. hydrophila* infections of fish and humans, particularly with regard to characterization of serotypes of virulent strains.

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

A panel of 70 *A. hydrophila* isolates from Australian freshwater and imported ornamental fish

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). Only one isolate (86:5879-G), of four isolates obtained from different barramundi from the same region and on the same date, reacted with F26P5C8, indicating the heterogeneous nature of *A. hydrophila* populations, even in a localized area. A very weak cross-reactivity was first observed with an *A. sobria* isolate by indirect immunofluorescence, but was eliminated following Western blot analysis of whole cell lysates (results not shown).

These studies of Australian isolates show that some isolates were capable of being detected with F26P5C8 and have epitopes cross-reactive with virulent EUS isolates from Malaysia and the Philippines. However, the virulence status of the positive Australian isolates has not been determined.

Characterization of surface antigen

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

The activity of F26P5C8 in Western blots was such that the supernatant obtained from hybridoma culture resulted in intensely stained blots with little detail. As a result, considerable difficulty was encountered 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 45 kDa and consistent with the antigen being LPS O polysaccharide (Janda 1991). Despite the diverse geographic and host range of fish from which the isolates were obtained, there was a degree of similarity in the patterns observed. All the isolates displayed some 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 are thought to be closely related.

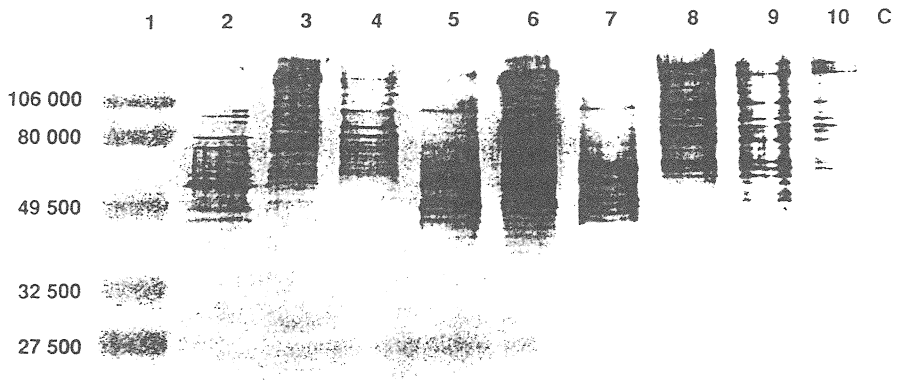

Figure 1. Immunoblotting of lysates, prepared from *A. hydrophila* isolates held at the Australian Fish Health Reference Laboratory, with F26P5C8 antibodies. Prestained molecular weight standards (Bio-Rad) were run on the lane 1. Lysate samples of isolates were loaded and run on lanes 2–10, in the same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

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 2 h. All were positive in indirect immunofluorescence tests, whereas isolates Ah86 and Ah138, which acted as controls, were negative. These results indicated that the antigenic determinant was heat stable and LPS rather than protein.

Protein digestion of extracted LPS and immunoblotting provided additional evidence that the surface antigen recognized by F26P5C8 antibodies was LPS. Immunoblotting of LPS extracted from *A. hydrophila* isolates that were positive (85:584-1A) and negative (85:8438) in reactivity with F26P5C8 showed similar results following proteinase K treatment of samples (Fig. 2). Profiles of these immunoblots were consistent in molecular sizes to those obtained for immunoblots of the cell lysates. The data indicate that the suface determinant of F26P5C8 antibodies was not the S-layer surface protein isolated and biochemically characterized by Dooley, McCubbin, Kay & Trust (1988). Instead, the bands of 45–65 kDa produced in the immunoblots are similar to the O side-chain core oligosaccharides identified by SDS-PAGE of proteinase K-treated LPS from *A. salmonicida* (Chart, Shaw, Ishiguro & Trust 1984).

Conclusions

The most virulent strains of *A. hydrophila* associated with EUS have been shown to belong to serotype I (Torres *et al.* 1992). While F26P5C8 antibodies recognize all serotype I isolates, and in particular, O side-chain core oligosaccharides, there were some reactions with other isolates indicating the need for clarification on serotyping. The detection of virulent serotype I isolates

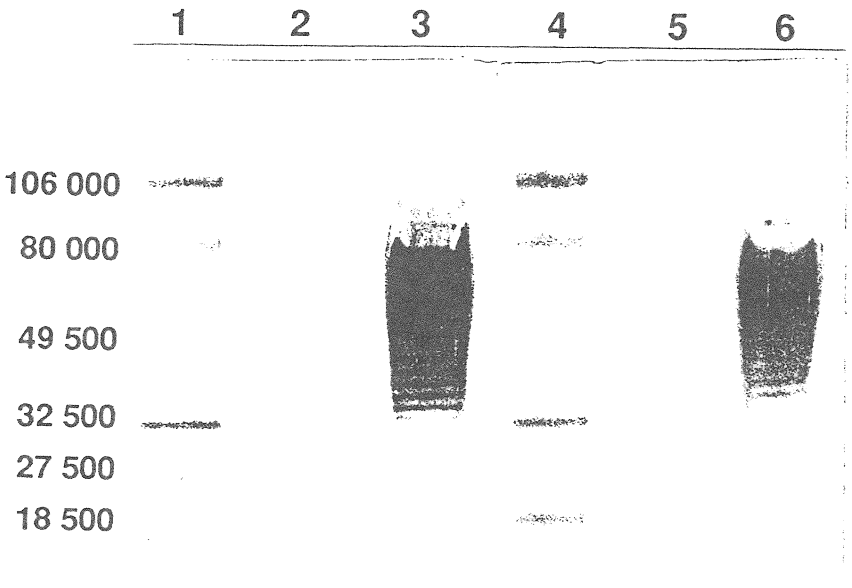

Figure 2. Immunoblotting of LPS extracted from *A. hydrophila* isolates that were positive (85:584-1A) and negative (85:8438) in reactivity with F26P5C8 antibodies in indirect immunofluorescence and ELISA tests. Lanes: 1 and 4, low molecular weight pre-stained standards (Bio-Rad); 3 and 6, 85:584-1A LPS; 2 and 5, 85:8438 LPS. LPS in lanes 5 and 6 was subject to protein digestion with 0·25 mg ml^{-1} of proteinase K for 15 min at 37 °C before immunoblotting.

by F26P5C8 antibodies, using either ELISA or FITC-immunofluorescence, provides a basis for further studies on the serological characterization and virulence of *A. hydrophila* isolates.

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Attachment of *Vibrio* pathogens to cells of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Abstract. The attachment of *Vibrio* pathogens to cells of rainbow trout, *Oncorhynchus mykiss* (Walbaum), was studied by use of species-specific monoclonal antibodies and indirect FITC-immunofluorescence microscopy. *Vibrio anguillarum*, *V. ordalii* and *V. parahaemolyticus* attached to cultured cells of rainbow trout gonads, various tissues of cryostat sections of whole fish, and smears of gills, intestine, buccal mucosa and skin. The attachment was inhibited by prior incubation of bacteria with monoclonal antibodies at titres of 1:32, or less. Other *Vibrio* pathogens used in this study did not attach to any trout cells. The research provides approaches to study the mechanisms of bacterial attachment in the onset of *Vibrio* infections.

Introduction

Vibriosis induced by the marine bacterium *Vibrio anguillarum* was first described in an outbreak of disease in eels from the Baltic Sea by Bergman in 1909. This disease was characterized by the appearance of bloody lesions in the musculature of the infected fish (Pacha & Kiehn 1969). A number of vibrios have now been reported to be associated with fish infections (Austin & Austin 1987). Amongst these, *V. anguillarum* and *V. ordalii* were reported as important pathogens of fish (Evelyn 1971; Schiewe, Trust & Crosa 1981; Egidius, Wiik, Andersen, Hoff & Hjeltnes 1986).

Adherence of *V. cholerae* O1, non-O1 and *V. parahaemolyticus* to human and rabbit small intestine has been observed *in vitro* (Pierce, Kaper, Mekalanos & Cray 1985; Teppema, Guince, Ibrahim, Paques & Ruitenberg 1987; Yamamoto & Yokota 1988, 1989; Yamamoto, Kamano, Uchimura, Iwanaga & Yokota 1988; Nakasone & Iwanaga 1990). It was believed that adherence of *V. cholerae* to intestinal mucosa was a necessary step in the pathogenesis of cholera (Pierce *et al.* 1985), although it was considered as a noninvasive bacterium.

This paper reports on evidence that the fish pathogens *V. anguillarum*, *V. ordalii* and *V. parahaemolyticus* attach to different tissues of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and that monoclonal antibodies specific to the pathogens inhibit attachment of the bacteria.

Materials and methods

Bacterial sources and culture

Vibrio anguillarum AFHRL 1 was obtained from the Australian Fish Health Reference Laboratory, CSIRO, Geelong, Australia. *V. anguillarum* serotypes O1 to O10 from the Institute of Hygiene and Microbiology, Royal Veterinary and Agricultural University, Denmark, and

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V. anguillarum MPL from Mt Pleasant Laboratory, Department of Agriculture, Launceston, Tasmania, Australia. Strains *V. alginolyticus* ACMM 101, *V. carchariae* ATCC 35084, *V. damsela* ATCC 33537, *V. harveyi* ACMM 130 and *V. ordalii* ATCC 33509 were obtained from the Australian Collection of Marine Micro-organisms at the Sir George Fisher Centre for Tropical Marine Studies, James Cook University of North Queensland, Townsville. *Vibrio cholerae* 569B Inaba, *V. cholerae* non-O1 V3, *V. parahaemolyticus* FC1011 and *V. vulnificus* were obtained from the Karolinska Institute, Department of Clinical Bacteriology, Huddinge University Hospital, Sweden. Although *V. salmonicida* has been shown to be a pathogen of fish (Egidius *et al.* 1986), it was unavailable for use in this study.

All *Vibrio* strains were grown in luminous medium, developed from the basic medium of Reichelt & Baumann (1973) by the addition of $5\,g\,l^{-1}$ tryptone and $5\,g\,l^{-1}$ yeast extract.

Cultivation of trout cells

A rainbow trout gonad cell-line (RTG2) was supplied by the Australian Fish Health Reference Laboratory, CSIRO, Geelong. Cultures were grown in minimum essential Eagle's medium (Cytosystems) at 22°C with 3% CO_2.

In preparing cultured RTG2 cells for immunofluorescence tests, the culture medium was discarded from a culture flask and the remaining cell monolayer washed with 50 mM PBS at pH 7·4. The cell monolayer was then removed by the addition of 5 ml of 0·25% (w/v) trypsin (Cytosystems) in PBS at pH 7·4, for 2 min. The trypsinized cells were centrifuged for 3 min at 100 *g* and then resuspended at a density of 0·1 million cells ml^{-1} in pre-warmed fresh medium. A 50 μl sample of the cell suspension was placed onto a clean sterile 22 × 22 mm coverslip (Mediglass No. 1) and placed in a sterile petri dish. After 15 min, 5 ml of pre-warmed fresh medium was gently added. The cells were then incubated at 22°C with 3% CO_2 to produce a near confluent layer after 5 days.

Preparation of cell smears and cross sections of juvenile trout

Rainbow trout of approximately 3 cm in length were stunned and then immediately frozen at −70°C. In preparing smears of cells from different tissues, frozen fish were firstly rinsed with 0·9% (w/v) NaCl and then soaked for 15 min in 0·9% (w/v) NaCl containing $200\,\mu g\,ml^{-1}$ streptomycin (Glaxo). Skin, gills, muscle, buccal mucosa and gut cells were smeared onto clean slides, and after airdrying, fixed in 2% (v/v) formalin (BDH) at room temperature for 15 min. The slide preparations were stored at −70°C until required.

Cross-sections of whole fish were prepared from juveniles stored for up to 2 weeks at −70°C. The fish were transferred to liquid nitrogen for 10 min prior to sectioning. Frozen sections of 10 μm thickness were then cut using a Cryocut E cryostat (Reichert Jung) and placed on clean slides. The sections were fixed in 2% formalin and then stored at −70°C until used in immunofluorescence tests. Additional cross sections of whole fish were double stained with haematoxylin and eosin (H&E) for use in the identification of tissues.

Monoclonal antibodies

Monoclonal antibodies had been developed in this laboratory against a wide range of *Vibrio* sp. pathogenic to humans and fish (Hanna, Altmann, Chen, Smith, Cosic, Moon & Hammond 1992). The MAbs used in this study, included F13P13F, F18P66C, F6P55C, F15P12B, F24P56G,

F23P11C, F12P411E, F31P46F and F7P511C which reacted with *V. anguillarum*, *V. ordalii*, *V. parahaemolyticus V. alginolyticus*, *V. carchariae*, *V. damsela*, *V. harveyi*, *V. vulnificus* and *V. cholerae* O1, respectively. Another MAb, F11P411F, that reacted with only bacterial species of the genus *Vibrio*, was used in this study to identify *V. cholerae* non-O1 and *V. anguillarum* serotypes O2 to O10 for which there were no specific MAbs available. Antigenic determinants recognized by the MAbs were all heat-stable and considered to be lipopolysaccharide (LPS).

Attachment of vibrios to cultured cells, smears and sections

Fish cells grown on coverslips were fixed in 2% (v/v) formalin and then washed twice in PBS. A 100 μl *Vibrio* suspension containing 1×10^8 cells ml^{-1} at the stationary phase was added to the fixed fish cells and incubated at room temperature for 45 min. After three washes with PBS, a monoclonal antibody specific to the bacteria was added to the fish cells and then incubated at room temperature for 45 min. After another three washes in PBS, 100 μl of 1:40 goat anti-mouse FITC-conjugate (Silenus) was added to the cells for 45 min, at room temperature. After a further three washes in PBS, the cells were mounted in PBS containing 90% (v/v) glycerol and 4% (v/v) propyl gallate. The attachment of vibrios to the fish cells was initially examined under phase contrast and then FITC-immunofluorescence microscopy using a Zeiss epifluorescence system.

The procedures for studying attachment of bacteria to smears of trout tissues and cryostat sections of trout were the same as those followed for cultured trout cells. Controls followed the same procedures except that bacteria were not included in one control and several isotypic MAbs were used in others.

Inhibition of Vibrio *attachment by species-specific monoclonal antibodies*

Inhibition tests were based on the procedures for studying *Vibrio* attachment. Each test was carried out by initially mixing 1 ml of a 1×10^8 cells ml^{-1} stationary phase *Vibrio* suspension with 1 ml of supernatant, containing a MAb that recognized only the *Vibrio* sp. being tested. The mixture was incubated at room temperature for 45 min, then centrifuged at 13 000 rpm for 1 min in a bench centrifuge (MSE, Micro Centuar). The supernatant was discarded and the pelleted bacteria were washed three times with PBS before they were resuspended in 2 ml of PBS. The *Vibrio* suspension was then poured onto the fish cells and incubated at room temperature for 45 min. After another three washes with PBS, 100 μl of MAb supernatant was added to the fish cells and incubated at room temperature for 45 min. Following three washes with PBS, 100 μl of 1:40 goat anti-mouse FITC-conjugate was added and the fish cells incubated for 45 min at room temperature. After further washes, the cells were mounted in PBS containing 90% (v/v) glycerol and 4% (v/v) propyl gallate, and then examined by FITC-immunofluorescence microscopy.

In order to determine the effective titre of MAb for inhibition of bacterial attachment, the MAb supernatant was diluted with PBS to 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. A 100 μl aliquot of each diluted MAb was mixed with an equal volume of 1×10^8 stationary phase *Vibrio* suspension and then incubated at room temperature for 45 min. Each mixture was added to a 75% confluent layer of cultured trout cells and incubated at room temperature for 45 min. After appropriate washes and incubation with goat anti-mouse FITC-conjugate, the cells were examined by epifluorescence microscopy.

A cross inhibition test between different species was carried out by mixing a 200 μl volume

of *V. anguillarum*, containing 1×10^8 cells, with an equal volume of undiluted MAb, F18P66C, which was specific to *V. ordalii*. After 45 min incubation at room temperature, the mixture was poured onto coverslips, 75% confluent with cultured RTG2 cells, for 45 min at room temperature. Following three washes with PBS, 100 μl of undiluted monoclonal antibody, F13P11F, specific to *V. anguillarum*, was added to the cells. After another three washes, 100 μl of goat anti-mouse FITC conjugate, diluted 1:40, was added for 45 min at room temperature before the preparations were mounted and examined by epifluorescence microscopy.

Results and discussion

The three *Vibrio* species, *V. anguillarum*, *V. ordalii* and *V. parahaemolyticus*, were found to attach to fish cells (Table 1). Of the *V. anguillarum* strains it was found that only serotypes O1, O2, O4 and O8 attached to fish cells, but of these serotypes O1 and O2 showed the greatest attachment. It has been reported that *V. anguillarum* serogroup O1 strains are the most pathogenic of 10 serogroups, with over 70% of all *Vibrio* infections being of serogroup O1 and 15% being O2 (Sorensen & Larsen 1986). Similarly, Myhr, Larsen, Lillehaug, Gudding, Heum & Hastein (1991) reported that the great majority of vibrios recovered from Norwegian marine farmed fish were *V. anguillarum* serogroups O1 and O2. The other *Vibrio* species did not attach to the trout cell preparations under the experimental conditions.

Vibrio *attachment to cultured rainbow trout cells*

The cultured RTG2 rainbow trout cells were clearly identified under phase contrast microscopy (Fig. 1a). Evidence for the attachment of both *V. anguillarum*, *V. ordalii* and *V. parahaemolyticus* to the surface of the RTG2 cells was indicated by the strong FITC-immunofluorescence of attached bacteria (Fig. 1b). Controls showed no FITC-immunofluorescence and it appeared that the other *Vibrio* strains did not adhere to the RTG2 cells.

Vibrio *attachment to cells from smears of rainbow trout tissues*

Vibrio anguillarum, *V. ordalii* and *V. parahaemolyticus* attached to cells from smears of several tissues, including skin, gills, buccal mucosa and gut. Figure 1c of a skin smear shows an example of scales seen under phase contrast microscopy. Strong FITC-immunofluorescence of *V. anguillarum* adhering to the scales is shown in Fig. 1d. There was no evidence to indicate that the other *Vibrio* strains attached to the cellular smears of the skin or other tissues.

Vibrio *attachment to cryostat sections of rainbow trout*

Figure 1e shows part of a cryostat cross-section through the mid-body region of a rainbow trout as seen under phase contrast. In general, the FITC-immunofluorescence of attached bacteria was localized to the skin and not the underlying muscles (Fig. 1f). *Vibrio anguillarum*, *V. ordalii* and *V. parahaemolyticus* showed positive attachment, but the other *Vibrio* species did not.

Inhibition of Vibrio *attachment*

Attachment of *V. anguillarum*, *V. ordalii* and *V. parahaemolyticus* to fish cells was inhibited by MAbs specific to each bacterial species. Titres of MAb, ranging from undiluted supernatant

Table 1. Attachment of *Vibrio* pathogens to rainbow trout tissues

	Attachment of rainbow trout tissues*		
Vibrio strains	Cultured cells	Smears	Cross sections
V. alginolyticus ACMM 101	–	–	–
V. anguillarum AFHRL 1	+++	+++	+++
V. anguillarum MPL	+++	+++	+++
V. anguillarum O1	+++	+++	+++
V. anguillarum O2	+++	+++	+++
V. anguillarum O3	–	–	–
V. anguillarum O4	++	++	++
V. anguillarum O5	–	–	–
V. anguillarum O6	–	–	–
V. anguillarum O7	–	–	–
V. anguillarum O8	–	+	+
V. anguillarum O9	–	–	–
V. anguillarum O10	–	–	–
V. carchariae ATCC 35084	–	–	–
V. cholerae O1 Inaba 569B	–	–	–
V. cholerae non-O1 V3	–	–	–
V. damsela ATCC 33537	–	–	–
V. harveyi ACMM 130	–	–	–
V. ordalii ATCC 33509	+++	+++	+++
V. parahaemolyticus FC1011	++	++	++
V. vulnificus (Sweden)	–	–	–

* Attachment: +++, very strong; ++, strong; +, weak.

to 1:32, completely inhibited bacterial attachment. Monoclonal antibodies diluted 1:64, or greater, did not appear to inhibit bacterial adherence.

When *V. anguillarum* strain AFHRL 1 or serotype O1 was initially mixed with undiluted MAb F18P66C, specific to *V. ordalii* ATCC 33509, the *V. anguillarum* bacteria were found to attach to fish cells and were detected by MAb F13P13F, specific to *V. anguillarum*. However, neither strain of *V. anguillarum* attached to the fish cells if initially mixed with MAb F13P13F. This means that the MAb specific to *V. ordalii* did not inhibit *V. anguillarum* bacterial attachment. In a revised experiment it was found that *V. ordalii* was also not neutralized by the *V. anguillarum* specific MAb. These data indicate that the blocking of the *Vibrio* surface epitopes by diagnostic MAb prevents attachment of the vibrios to fish cells.

In a manner similar to the attachment of *V. cholerae* to human small intestinal mucosa, the attachment of *V. anguillarum*, *V. ordalii* and *V. parahaemolyticus* to fish cells is a likely first step of infection. There is evidence that *V. parahaemolyticus* can attach to gastropod cells *in vitro* (Kumazawa, Okamoto & Kato 1990). Further support is shown by the current findings in which the attachment of *V. anguillarum* serotypes O1 and O2 were greater than the other serotypes and both strains have previously been shown to be the most virulent serotypes (Sorensen & Larsen 1986; Myhr *et al.* 1991). The underlying reasons for the strong attachment of serotypes O1 and O2 are unexplained. The present authors are currently isolating surface components of pathogenic *Vibrio* strains to determine those components involved in the attachment process.

The current research provides for further studies regarding the nature of bacterial receptor

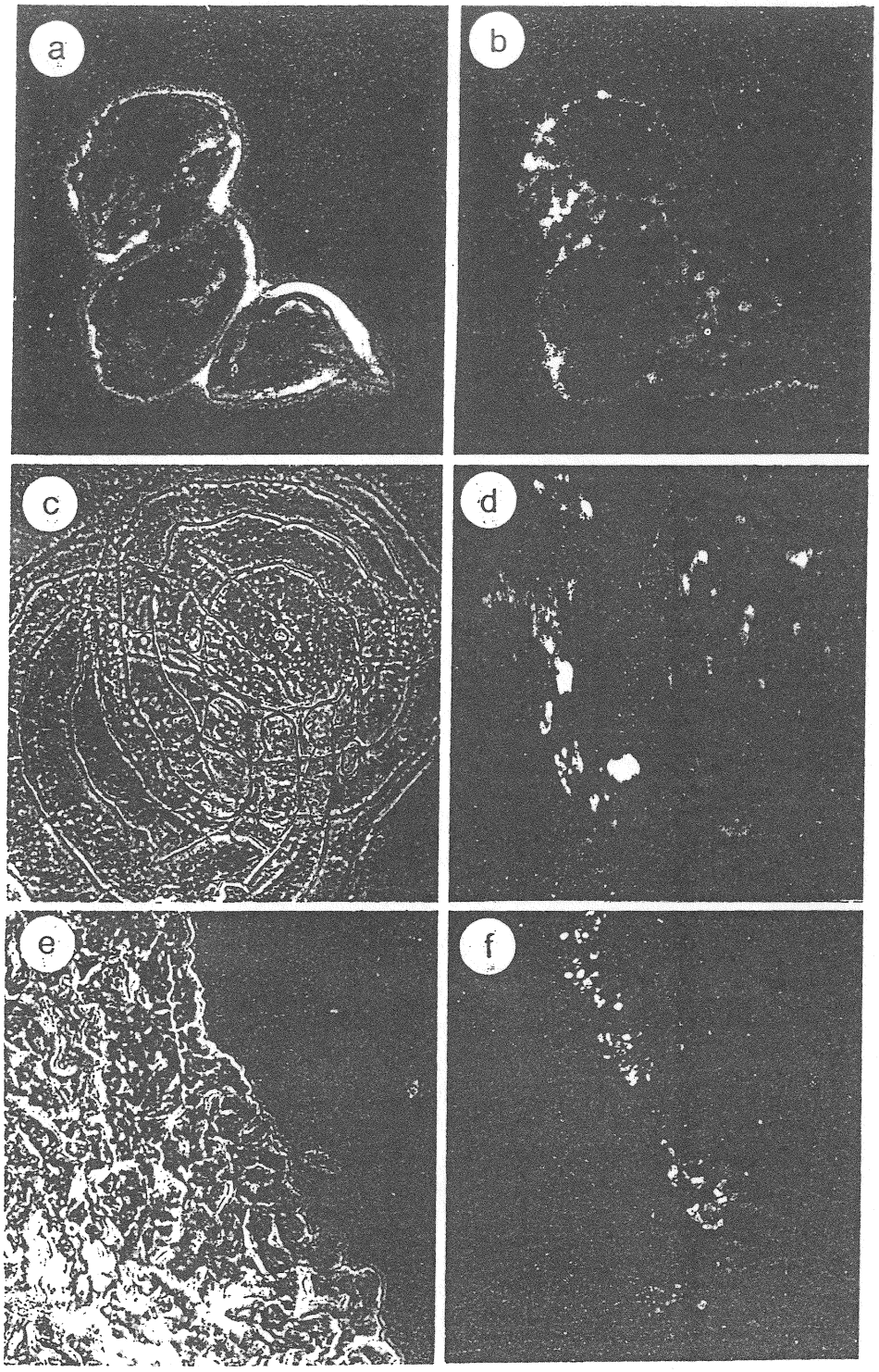

Figure 1. Attachment of *V. anguillarum* to rainbow trout cells: (a & b) cultured RTG2 gonad cells, ×400; (c & d) scales from a skin smear, ×100; (e & f) cryostat sections of skin through the mid-body, ×100. Photomicrographs a, c and e are phase contrast, whereas b, d and f are indirect FITC-immunofluorescence using a *V. anguillarum* diagnostic MAb F13P13F as the primary antibody

sites on host cells. Research of this type should include the use of a wide range of different cell-lines with known *Vibrio* pathogens to determine their interactions.

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