Development of Vaccines and Diagnostic Monoclonal Antibodies against Bacteria Associated with Diseases of Wild and Cultured Finfish and Shellfish

Project 93/130

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& Chemical Sciences

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FISHERIES
RESEARCH &
DEVELOPMENT
CORPORATION

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Non-technical Summary

A number of bacterial species are well known for as primary pathogens of marine fish and shell-fish and several others have been implicated with diseases. Overall, these bacteria have resulted in substantial economic losses, particularly in farmed aquatic animals. Austin and Austin (1993) cited *Vibrio* species as being the scourge of marine fish and shellfish, causing annual losses in Japan at that time of over $30 million. Therefore, there has been an increasing demand for the tests that can quickly identify potential pathogens in water, or fish and shellfish, be it live, dead or processed products.

In order to rapidly identify potential disease causing bacteria we set out to produce a library of monoclonal antibodies that could be used in diagnostic tests. Monoclonal antibodies (mAbs) are now used widely in medical and veterinary situations, and are produced by a methodology developed by Kohler and Milstein (1975), through which they were awarded Nobel prizes. The basic terminology and protocols for producing mAbs are as follows. When an animal such as a mouse is injected with foreign materials (=antigen) such as killed bacteria, which are infectious to fish and shell-fish, the usual response is a proliferation of a number of different antibody-producing cells, 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 we used for identification of antibody production and subsequent screening for bacteria, 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 can be frozen for later use when antibody supplies require replenishing

In regard to preparation of bacteria for production of mAbs, we had previously developed a novel technique to extract a chemical component of a bacterial cell surface (i.e. lipopolysaccharide, termed LPS), which was unique to that bacteria. This component conveyed bacterial identity via mAbs produced to it, at either the species or genus taxonomic level.

As a result, we have now produced an extensive library of diagnostic mAbs, many of which have been used in the identification of bacteria associated with major 'kills' of aquatic fish and shellfish, including hatchery-reared juvenile oysters, scallops, clams, prawns and fish. Through this testing, some hatchery management techniques have been altered leading to successful production, even though some of the original 'kills' were substantial, causing considerable losses in economic terms. A workshop, held at Deakin University in June 1995, demonstrated diagnostic techniques utilising the mAb technology. The mAbs are currently available for purchase from TECRA Diagnostics in Sydney (see Appendix I).

Our findings that many of the antibodies produced against injected bacterial LPS components identified bacteria within a genus, provided an excellent opportunity to test whether the components could act as generic vaccines. Therefore, as a part of the research we extracted LPS components from *Vibrio* and *Aeromonas* species that were not pathogens of fish and used them to immunise rainbow trout and goldfish, respectively, by intra-peritoneal injections. Subsequent challenges with pathogenic strains of *Vibrio anguillarum* and *Aeromonas salmonicida*, indicated cross-protection to the pathogens. A further test using an oral route of immunisation with goldfish showed protection against infection by *Aeromonas salmonicida.* Hitherto, oral immunisations have had varied success. Overall, the results indicated that the LPS components had potential as vaccines, but large-scale extraction for commercial use would likely be a costly exercise. Chemical synthesis, as an alternative, would appear to be the best route of production, but would require prior analyses of the components and subsequent development of production methods. However, once accomplished, a wide range of important vaccines could be produced.

Background

In the past several years, we have developed an extensive library of diagnostic monoclonal antibodies (mAbs) against pathogenic vibrios which cause diseases in fish and shellfish (Hanna *et al.*, 1992; Chen *et al.*, 1992). Vibrios used to make mAbs included *V. alginolyticus, V. anguillarum, V. carchariae, V. cholerae* O1 and non-O1, *V. damsela, V. furnissii, V. harveyi, V. ordalii, V. parahaemolyticus* and *V. vulnificus*. Overall, 102 mAbs were developed, a number of which have been successfully used in the identification of *Vibrio* isolates, as they were selected for strain, species and genus specificity. These mAbs have already been useful in diagnostic, taxonomic and research studies. For example, the species-specific mAbs were found to inhibit the attachment of *Vibrio* pathogens to susceptible host cells (Chen and Hanna, 1992) and the genus-specific mAbs have shown that *V. damsela* should be relegated out of the genus. During the past six months of a previous FDRC grant we have also successfully produced mAbs that identify the fish pathogens *V. salmonicida, V. spendidus* and *V. tubiashii*.

The following intellectual property is covered by Australian Petty Patent No. 660268 "Bacteria-Specific Lipopolysaccharide-Like Molecules" and any research utilising the intellectual property requires approval.

Additional independent research at Deakin University has led to a very important finding concerning the nature of the antigen responsible for species-specificity of mAbs identifying gram-negative bacteria. Genus-specific mAbs react with LPS fragments of extracted LPS, and in the case of *Vibrio* species, the smallest sized fragment was always 18.5kDa, no matter what the species the LPS came from. As a consequence of these research findings the 18.5kDa LPS of several *Vibrio* species was isolated and used to immunise mice. The mAbs produced from these mice were either species-specific or genus-specific, thereby being very good for diagnostic purposes.

It was rather surprising to find that these diagnostic mAbs reacted with 18.5kDa LPS fragment size of total LPS, live bacteria, and bacteria killed by heat, formalin or sodium azide, indicating that the antigen was exposed on the surface of the bacteria. Subsequently, other gram-negative bacteria were found to also possess LPS fragments of approximately similar size and these have been used to produce genus-specific polyclonal antibodies. Overall, the data indicated that fragments from any gram-negative pathogen could be used to produce diagnostic mAbs for identification purposes.

Another potential use of the LPS fragments is in the production vaccines. The findings that the fragments could produce genus-specific mAbs indicated a possible use of the fragments to induce cross-protective antibodies against a number of pathogenic species in the same genus. Previously, many vaccines have relied on growing bacteria and killing the cells prior to use as a bacterin vaccine.

Need

The project had two major components that addressed different needs. They were:

(1) The rapid identification of gram-negative pathogens in disease situations. This need had been emphasised by Austin (1987) who stated that:

> "The most common shortcomings in diagnosis of fish diseases concern the identification of bacterial isolates. There are two schools of thought, namely those who rely on serology and those who rely on phenotypic tests. Concerning the former, we will preface further discussion by wholeheartedly endorsing a view that reliable diagnoses occur only with monospecific antisera to assure the homologous reaction between antigen and antibody. Most diagnosticians use polyclonal antisera raised in rabbits. The extent of any cross-reactions has not been adequately determined. Clearly, there is an urgent need for the highly specific monoclonal antibodies."

We had already started on this path and at the beginning of the project had the best panel of *Vibrio* and *Aeromonas* diagnostic mAbs in the world. The new diagnostics would be produced by extracting LPS fragments from additional important gram-negative fish and shellfish pathogens and using them to produce diagnostic mAbs. In order of priority, diagnostic mAbs were produced against the bacteria: *Yersinia ruckeri, Pseudomonas anguillseptica, P. fluorescens, Vibrio mimicus* (due to several recent diseases incidences in Australia and overseas), *Flexibacter columnaris, Edwardsiella ictaluri, E. tarda (E. anguillimortifera).*

(2) To test the effectiveness of the fragments in producing a genus-specific vaccines. In regard to vaccine production we already had data from a preliminary trial that immunization with LPS fragments from one pathogenic *Vibrio* species conveyed protection to others in the same genus. Continuation of this research would form the important second part of the project. Although there existed a need for a *Vibrio* vaccine,

there was also a need for protection against another important gram-negative pathogen, namely *Aeromonas salmonicida.* It had been clearly stated by Austin (1987) that:

> "The development of an effective vaccine against the rigours of *Aeromonas salmonicida* remains one of the great challenges to researchers."

This need would be addressed by utilizing *Aeromonas* LPS fragments to induce protection against *A. salmonicida* infection in fish.

Objectives

1. Identification and characterization of species-specific antigenic determinants that are heat-stable components of the lipopolysaccharide (LPS) on pathogenic gram-negative bacteria;
2. Prepare a library of species and genus-specific monoclonal antibodies against the heat-stable LPS components of pathogenic gram-negative bacteria;
3. Select appropriate hybridomas, that produce high affinity monoclonal antibodies, for transfer to strategic companies;
4. Complete the testing of specific LPS fragments extracted from one *Vibrio* species to produce a vaccine against all *Vibrio* pathogens;
5. Use the same approach as in (4) to test the genus-specific protection afforded by similar LPS fragments extracted from additional pathogens, particularly *Aeromonas salmonicida*; and
6. Develop and market commercial products for use in rapid diagnoses, and research, of infections of wild and cultured fish and shellfish.

Methods

Detailed descriptions of the methods employed in the production of diagnostics are contained in a paper given in Appendix I. The project was divided into three main parts, and included:

- Preparation of bacterial antigens;
- Use of bacterial antigens to produce diagnostic monoclonal antibodies; and
- Use of bacterial antigens as vaccines.

Preparation of bacterial antigens

Bacterial strains and culture

Although a large number of gram-negative bacterial strains were held in stock, additional pathogenic gram-negative strains were needed for testing, and were preferably acquired as American Type Culture Collection (ATCC) strains. The bacterial strains were grown on appropriate agar media and subcultured bimonthly, as well as being stored at -80°C with 33%(v/v) glycerol. In order to obtain large quantities of bacteria for the testing of mAbs, or extraction of LPS, bacterial strains were grown in 20 L of appropriate liquid broth and then collected by centrifugation at 10,000 g for 20 min. After washing three times in 0.01 M phosphate-buffered saline (PBS) at pH 7.4, they were killed in 1.0% sodium azide in PBS and stored at 4°C.

Extraction of total LPS

Total LPS was extracted by the hot water-phenol, according to the method of Westphal *et al.* (1952). To maximise yields of extracted LPS, the phenol-layer was extracted twice with an equal volume of distilled water and the combined aqueous phases dialyzed extensively against distilled water. The total LPS was subjected to repeated ultracentrifugations at 102,000 g for 4 h at 4°C to remove contaminating proteins and

nucleic acids. The purity of LPS was monitored by UV spectroscopy at a wavelength of 190-350 nm (Westphal *et al.*, 1983).

Separation of LPS components

Total LPS was separated, together with pre-stained molecular weight markers, by SDS-PAGE, using 12.5% acrylamide gels (Laemmli, 1970) and bands visualized by silver staining (Hitchcock and Brown, 1983). LPS components were then cut from replicate unstained gels and LPS components extracted in double distilled water (DDW), followed by dialysis against DDW water to remove free acrylamide. The LPS components were freeze-dried in pre-weighed tubes to determine amounts isolated and then dissolved in DDW to make up final concentrations of 1 mg/mL.

Use of bacterial antigens to produce diagnostic monoclonal antibodies

Immunization of mice with whole cells or LPS components

The general methods to be used for the production of monoclonal antibodies have been described by Goding (1980, 1985). Separate female BALB/c mice were given an injection of approximately 10^7 of killed washed cells, or 1.0-2.5 mg/mouse of LPS, mixed with equal volume of Freund's complete adjuvant (CSL), and after one week another dose of antigen with an equal volume of Freund's incomplete adjuvant. If polyclonal antiserum titres were 1:1,000, or greater, in alkaline phosphatase ELISA (see later section), a final injection of antigen, without adjuvant, was given and spleens removed 4 days later.

Monoclonal antibody (mAb) production

Splenocytes were isolated and cell fusions made with mouse myeloma cells using polyethylene glycol (PEG-4000). Selection of hybrid cells was carried out in HAT culture medium, comprising RPMI-1640 medium with 20% (v/v) foetal calf serum plus hypoxanthine, aminopterin and thymidine. All hybridomas positive in first screenings (see next section) were transferred to 24-well plates and grown for about 2 days before being

screened in heterologous ELISA. Useful hybridomas were cloned twice by the limiting dilution technique (Goding, 1980), frozen at -80^{o}C and then stored in liquid nitrogen.

Screening of mAbs for species-specificities and isotyping

Initial screenings of mAbs involved determination of those which reacted with the gram-negative bacteria used in the immunizations. The screenings utilised an alkaline phosphatase ELISA (Karlsson *et al.,* 1986), in which plates had been coated overnight with bacteria. Heterologous screenings against a wide range of gram-negative bacteria were then performed to confirm that the mAbs were able to identify bacterial species or genera. The results were confirmed by indirect immunofluorescence. Isotyping of mAbs was carried out using Bio-Rad isotyping kits to determine the heavy and light chains present in the antibodies.

Application of the mAbs in fisheries problems

The mAbs were used to determine the infectious agent in a number of situations, including diseased fish and shellfish, disease monitoring, water quality testing, and research. Techniques employed here were usually ELISA or immuno-fluorescence microscopy, and are detailed in papers published as outcomes of this project (see Publications section).

Use of bacterial antigens as vaccines

Fish and culture conditions

The fish used in the vaccine trials were obtained as homogenous groups from two sources. Rainbow trout (*Oncorhynchus mykiss* Walbaum) fingerlings were purchased from the Ballarat Trout Farm, Ballarat. Goldfish (*Carassius auratus*) of 5-6 cm length and of a non-diseased background and bred at Dixon's Creek, were kindly donated by R. Datodi, Pet and Aquarium Industries, Preston.

On arrival, fish were placed in tanks in the Deakin University aquarium room and allowed to acclimatise to the laboratory conditions over 1 week. All tanks were

constantly aerated. The room photoperiod was set at 12 h D: 12 h L, and room temperature set at 17°C. Only a few of the trout died in the first 1-3 days, presumably as a result of stress caused by transport. All goldfish acclimatised well. Rainbow trout were held in 1000 L Reln tanks and fed twice daily with trout pellets obtained from the Snobb's Creek Hatchery. Goldfish were held in 32 L Nally plastic tanks and fed flaked goldfish food. Tanks were covered with nylon mesh or plastic to prevent fish from escaping. Large black-plastic sheets were placed beneath and around tanks for containment of spilt water and subsequent decontamination, to prevent cross-contamination.

Water quality parameters of pH, nitrate concentration, ammonium concentration, were monitored. Water exchanges of about 1/3 total volumes with pre-conditioned fresh water, and vacuum-extraction of sediments, were carried out twice weekly.

Decontamination

All tanks and equipment utilised in challenges of fish with live bacteria were disinfected by soaking overnight in a chlorine bleach, followed by thorough rinsing with fresh water. Commercial bleaches containing 10-12% available free chlorine were added to give 3.5 mL/L. Challenged fish were handled using standard microbiological safety techniques, and following analyses, all items, including fish carcasses, were decontaminated by autoclaving.

Immunisations

LPS core components, for use as antigens, were exracted from *Vibrio anguillarum*, *Aeromonas salmonicida* and other *Aeromonas* species, using the same methods as for production of monoclonal antibodies in mice (see the earlier methods section). Previous tests had indicated that an interperitoneal (*i.p.*) injection of 0.1-0.5 µg LPS in 0.1 mL of PBS was sufficient to produce an immune response in the size of fish we used. All fish were anaesthetised with 50 mg of benzocaine in 1 L of water prior to injection with antigen.

In normal vaccine trials, groups of 10 trout or goldfish were injected *i.p.* with antigen, and an additional control group injected with PBS only. However, based on the successful results of these trials another trial was carried out to determine whether oral dose of LPS components gave protection. In this trial the same dose of antigen was given orally by syringe.

In another trial, we aimed to determine whether immunisation with LPS of one species of *Aeromonas* gave protection against another (e.g. the pathogen *A. salmonicida*). We tested for cross-protection of LPS extracted from *A. hydrophilla* and *A. sorbria,* against *A. salmonicida*, and used LPS from *A. salmonicida* as a positive control, and LPS from *V. anguillarum* and PBS as two negative controls.

Testing for an immune response

In some vaccine trials anaesthetised fish were bled from the caudal vein, prior to and at least one month following immunisation, to determine the presence of circulating antibodies. The serum antibody titres were determined by routine ELISA using whole bacteria, which were of the same strain used to isolate LPS for immunisations (see methods in the monoclonal antibody screening section).

Finally, challenges of immunised and non-immunised fish, with live bacteria were carried out to determine whether the animals had been actively immunised.

Challenges to determine protection

The bacteria used in these trials were *Vibrio anguillarum* serotype O1 and *Aeromonas salmonicida* 85:9370-A, obtained fron the Australian Animal Health Laboratory, CSIRO, Geelong. At least two passages were carried out in isolated fish to test and increase the pathogenicity of the bacteria. The optimum dose for challenges were then determined. Numbers of bacteria used in the challenges were estimated from an optical density (OD) of 0.2 at A_{620} equating to about $5x10^8$ cfu/mL. We had previously constructed standard curves of optical density against numbers of viable bacteria. Fish were normally challenged by *i.p.* injection of 0.1 mL of live passaged bacteria in PBS, but in one challenge the bacteria were given orally in PBS.

Challenged fish were monitored daily for clinical signs of toxicity or disease, including lethargy, loss of appetite, loss of balance, erratic swimming movements, lesions and reddening of tissues, scale loss and death. Fish showing severe clinical signs of disease, or stress, were euthanased by an overdose of anaesthetic, prior to laboratory examination. Any dead fish were immediately removed. Post-mortem microbiological tests were performed on different tissues for the presence of bacteria, and if positive, bacteria identification was confirmed by immunofluorescence.

Detailed Results

The detailed results are contained in two papers given in the appendices. In particular the results are divided into two main parts, and include:

- Use of bacterial antigens to produce diagnostic monoclonal antibodies; and
- Use of bacterial antigens as vaccines.

Use of bacterial antigens to produce diagnostic monoclonal antibodies (mAbs)

We have now established an extensive world-class library of mAbs with capabilities of identifying most of the known pathogenic gram-negative bacteria. A comprehensive selection of important mAbs is listed in Table 1, and are now available commercially through TECRA Diagnostics, 28 Barcoo St., Roseville, NSW 2069. There are many others not listed here, but have similar identifying properties.

Table 1. Monoclonal antibodies available for diagnostic use

Monoclonal Antibody Number	Bacteria Identified and Reactive Antigen
F29P5B8	*Aeromonas* species (LPS)
F29P3C2	*A. salmonicida* (LPS)
F19P5E12	*A. salmonicida* (A protein)
F26P5C8	*A. hydrophila* (LPS)
F47P4D12	*Flexibacter* species(LPS)
F46P6A2	*F. columnaris*
F47P7A3	*F. maritimus* (LPS)
F41P4F9	*Yersinia* species(LPS)
F45P4D5	*Y. ruckeri* (LPS)
F11P4F11	*Vibrio* species (LPS)
F13P1F3	*V. anguillarum* (LPS)
F15P1A7	*V. alginolyticus* (Protein)

F24P5G6	*V. carchariae* (LPS)
F7P5C11	*V. cholerae* (LPS)
F7P2A5	*V. cholerae* (LPS), O1 strains
F27P5H10	*V. cholerae* (LPS), non O1 strains
F12P4E11	*V. harveyi* (LPS)
F44P2E3	*V. mimicus* (LPS)
F18P6C6	*V. ordalii* (LPS)
F6P5C5	*V. parahaemolyticus* (LPS)
F35P3F12	*V. salmonicida* (LPS)
F36P3B10	*V. splendidus* (LPS)
F37P2B7	*V. tubiashii* (LPS)
F31P5B8	*V. vulnificus* (LPS)
F23P7E7	*V. damsela* (LPS), *Photobacterium damsela*
F51P4C4	*Pseudomonas* species (LPS)
F50P1B5	*P. fluorescens* (LPS
F51P1F2	*P. aeruginosa* (LPS)

Use of bacterial antigens as vaccines

LPS protection of rainbow trout (Oncorhynchus mykiss) against Vibrio anguillarum

In an earlier vaccine trial LPS core antigen from *Vibrio anguillarum* was used to immunise small rainbow trout from the Snob's Creek hatchery. This trial did show some protection by the LPS antigen to the disease vibriosis, caused by *V. anguillarum*, but indicated the need for testing virulence of laboratory-held bacterial isolates, many of which lose virulence through repeated *in vitro* culture. The plan was to repeat the trial, using a virulent strain of *V. anguillarum.*

A virulent strain of *Vibrio anguillarum* was obtained from the Australian Animal Health Laboratories, CSIRO, Geelong, and passaged in small trout purchased from the Ballarat Trout hatchery, Ballarat. Early clinical signs of diseased fish occurred within a

day of challenge and included loss of appetite, lethargy, loss of equilibrium typical reddish skin lesions, particularly at the site of *i.p.* challenge. The later stages of the vibriosis resulted in liquefaction of the internal organs, including rupture of the bowel adding coliforms to the peritoneal cavity. We identified *Vibrio anguillarum* in all fish showing disease signs, by post-mortem isolation from the spleen and other tissues.

In a separate trial, we used three groups of trout immunised with core LPS from *Vibrio anguillarum* (VAN), core LPS from *Vibrio alginolyticus* (VAL) and PBS (control). Doses were 0.5 µg/fish. We included the LPS from *Vibrio alginolyticus* to determine whether there was cross-protection against the highly virulent strain of *Vibrio anguillarum* that we had acquired. A challenge at 4 weeks post-immunisation showed a distinct effect in the control fish, as 50% died within 1 day and the remainder were moribund (Fig. 1). All controls had died within 42 h. However, immunised fish were protected (i.e. no deaths), and survived more than 2 weeks before being euthanased. However, fish immunised with the non-relevant *Vibrio* antigen (i.e. VAN-LPS) or the antigen from the challenge species (i.e. VAN-LPS), showed a delyed response to the virulent challenge. A comparison of the immunised fish is interesting as 20% of the VAL-LPS group survived 2 weeks, at which stage the trial was terminated. This was a better result than the VAN-LPS group immunised with LPS from the bacteria used in the challenge. However, we did not compensate for dose of immunising antigen with differences in size of fish, a feature that is relevant to immunisations of commercial trout.

There were two major outcomes of this trial. The first was the protection given by the low dose of immunising antigen (i.e. 0.5 µg/ fish) with high bacterial challenge. Secondly, the protection given by a non-relevant indicated that the core LPS could elicit protective antibodies to virulent species within the same genus, viz *Vibrio*. This is understandable as it is the basis of the production of genus-specific monoclonal antibodies. The use of one vaccine for all *Vibrio* types would prove invaluable in aquaculture.

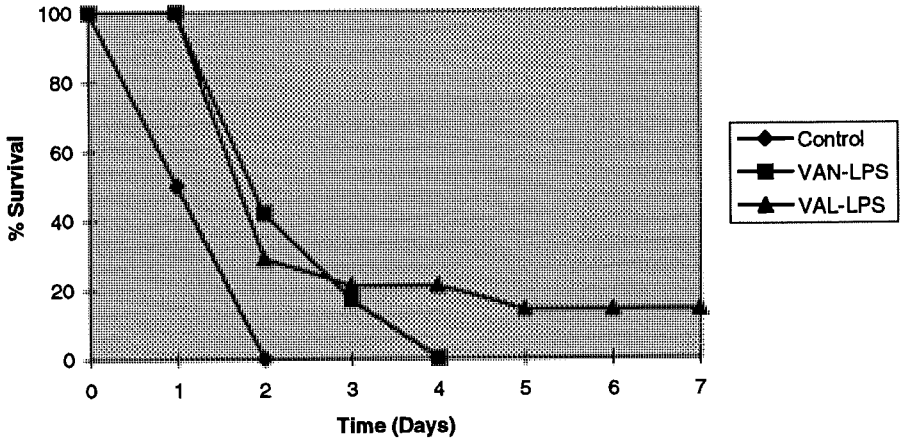

Figure 1. LPS protection of rainbow trout against *Vibrio anguillarum.*

LPS protection of goldfish (Carassius auratus) against Aeromonas salmonicida

A strain of *Aeromonas salmonicida* (85-9370-A) was obtained from the Australian Animal Health Laboratories, CSIRO, Geelong, and passaged twice in isolated goldfish. All goldfish used in these trials were kindly donated by the R. Datodi, Pet and Aquarium Industries. Early signs of diseased fish were loss of appetite, lethargy, loss of equilibrium, lesions at the point of *i.p.* challenge, and noticeable scale loss in patches. We identified *Aeromonas salmonicida* in all fish showing signs of disease, by post-mortem isolation from the spleen and other tissues.

In a first vaccine trial, 4 groups of goldfish were immunised with 1 μg of core LPS in 0.1 mL of PBS, and another control group injected with PBS only. After 47 days 2 immunised groups (i.e. replicates) were challenged with $5x10^5$ live bacteria (LD1&2), another 2 immunised groups (i.e. replicates) were challenged with $5x10^6$ live bacteria (HD1&2), and the control group challenged with $5x10^5$ live bacteria). We found that within 1 day of the challenge, all control fish had died, as well as some immunised fish (Fig. 2). However, a noticeable number of the immunised fish exhibited a quick recovery from the challenge and survived for a week, at which time the trial was terminated. There was only a marginal difference in survival of the fish challenged at the lower dose than

those at the higher dose. As with the trout trials, we did not compensate for dose of immunising antigen with differences in size of fish.

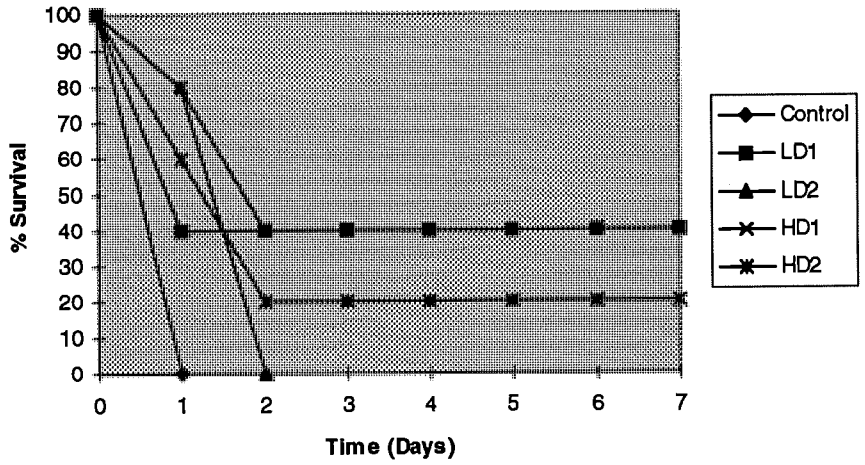

Figure 2. LPS protection of goldfish against *Aeromonas salmonicida.*

LPS cross-protection of goldfish (Carassius auratus) against Aeromonas species

We performed a test of cross-protective immunity against *Aeromonas salmonicida* by immunising groups of goldfish with 1 µg/fish of core LPS extracted from different species, viz *Aeromonas salmonicida* (ASA), *A. hydrophila* (AHY) and *A. sobria* (ASO). The control groups were immunised with PBS or non-relevant *Vibrio anguillarum* core LPS (VAN). We included the LPS from the additional *Aeromonas* species to determine whether there was cross-protection against the virulent strain of *Aeromonas salmonicida.*

Four weeks after immunisation, all groups of fish were challenged *i.p.* with live passaged *Aeromonas salmonicida* (85-9370-A) at a dose of $2xLD_{50}$. Within 48 h 75% of the control group and 78% of the group immunised with non-relevant *Vibrio* LPS had died. Only 3 fish of these two groups were alive 2 weeks later. However, all of the fish in the three groups immunised with LPS of different *Aeromonas* species were healthy at the end of 2 weeks when the trial was terminated.

This trial showed that fish immunised with LPS core components extracted from different species within the genus *Aeromonas*, such as *A. hydrophila* and *A. sobri*, produced cross protective immunity against infection by *Aeromonas salmonicida.*

Determination of circulating protective antibodies in goldfish (Carassius auratus) immunised against Aeromonas species

We repeated the last trial involving LPS cross-protection of goldfish (*Carassius auratus*) against *Aeromonas* species. This was done for three main reasons. Firstly, we needed to confirm the previous results. Secondly, we wanted to determine the amount of protective antibodies circulating in the blood of immunised fish. Lastly, we wanted to include an oral vaccine group to compare protection by this route of antigen administration with that of *i.p.* injection. Therefore, addition to the treatment groups used in the last trial we added one in which each fish was given an oral dose of 1.25 μg in 50 μL of PBS. Blood was taken from all fish at 30 days and 60 days post-immunisation and immediately frozen until ELISA end-point titres at dilutions of 1:50, 1:500, 1:1000 and 1:10,000 were carried out using cells of *Aeromonas salmonicida* as the coating antigen in the 96-well plates.

The results are shown in Table 2. They indicate clearly that the blood of fish immunised by *i.p.* injection or orally with any type of *Aeromonas* core LPS contained substantial amounts of protective antibodies against *Aeromonas salmonicida.* An important aspect of the results were the findings of significant amounts of antibodies present in blood at 60 days post-immunisation, particularly when the injected dose was only 0.1 μg per fish. Another important result was the production of significant amounts of circulating antibodies after oral immunisation. Both these results are really not surprising as we had observed them in mice during the production of monoclonal antibodies.

Table 2. Determination of circulating protective antibodies in goldfish immunised with core LPS

Fish Group	Type of Antigen	Route of Immunisation	Immunity based on ELISA results* (Post-immunisation)	
			At 30 days	At 60 days
1	Control (PBS only)	*i.p.*	-	-
2	Control (VAN-LPS)	*i.p.*	±	±
3	ASA-LPS	*i.p.*	+++	+++
4	AHY-LPS	*i.p.*	+++	+++
5	ASO-LPS	*i.p.*	+++	+++
6	ASA-LPS	oral	+++	+++

* the results indicated as +++ are for ELISA OD readings of >1.00 at dilutions of 1:500, or more, whereas - indicates the same as ELISA controls of BSA with OD readings of about 0.2 at any dilution, and the ± indicates a slight reaction (OD~0.4) at a dilution of 1:500.

The fish used to determine the presence circulating protective against *Aeromonas salmonicida* at 30 and 60 days post-immunisation were challenged with live *Aeromonas salmonicida* at 79 days post-immunisation. As expected from the blood analyses, all fish in the two control groups had died by the end of the following day. However, about 50% of the fish in the other 4 groups had also died after 1 day and were all dead after a further day. We believe that there was an incorrect dilution of bacteria and $>10^7$ bacteria had been administered in the challenges. This would account for the higher than expected mortalities.

Conclusions

Overall, the data indicates that the bacterial core LPS is effective in producing immunity to a pathogen, either by injectioon or orally. There is also good evidence that the LPS provides cross-protection against infection to other species within a genus.

Further research is now required to analyse the chemical structure of core LPS of different genera, with the aim that synthetic vaccines might one day be produced. Such vaccines would have wide implications in veterinary and medical situations.

Benefits

Various sectors of the industry will directly and indirectly benefit by the outcomes of the research. They include direct benefits to a wide range of aquaculture and wild fisheries, both professional and recreational, through protection and management of diseases in fish and shellfish. The benefit to the aquaculture industry sector is estimated to reduce production costs through rapid diagnoses of infectious agents. The benefit to rapid identification of disease in wild fisheries stocks, and in stocks of imported exotic fish held in quarantine, is also very important.

Commercialisation of the products will lead to benefits by all individuals or groups concerned with fish and shellfish health, including disease diagnosis, disease monitoring, and water quality testing , regardless of region or state.

Although we were unable to produce commercial vaccines to pathogens, such as the Vibrios and Aeromonads, we did show the potential of bacterial surface components to convey protection to infections. In particular, there was clear cross protection against infection within a genera of bacteria, and evidence of protection by oral administration. These features are important elements for future vaccine research. In particular, the development of an oral vaccine would have great commercial and aquaculture benefit.

Intellectual Property and Valuable Information

The intellectual property generated by this project is a number of hybridomas, now frozen, that secrete monoclonal antibodies in culture. In particular, these monoclonal antibodies can identify a range of fish and shellfish pathogens using standard immunological techniques, of which immunofluorescence microscopy is the most rapid, giving results in about 2 hours. Commercial availability of the mAbs is planned to be in two product lines, one product being 1-2 mL quantities for use in research laboratories, and the other is in an 'easy-use' diagnostic kit form. The kits will be developed when there is a market demand.

Deakin University and the FRDC have agreed to share the intellectual property ownership of R&D concerning the diagnostics, and have already established a commercial partner for the production, sales and marketing of products. The company is TECRA DIAGNOSTICS, whose business is operated within the facilities of Biotech Australia at the well-equipped laboratories and manufacturing site in Roseville, New South Wales (Address: PO Box 20, 28 Barcoo St., Roseville NSW, Australia). It produces kits, approved to the ISO9002 standard, for the rapid detection of pathogens and toxins. These are marketed and sold by Bioenterprises Pty Ltd, which is part of Biotech Australia Pty Ltd, Australia's largest biotechnology company. Both companies have their head office in Sydney and are owned by Hoechst Australia. TECRA DIAGNOSTICS is also doing collaborative research with Deakin University in producing diagnostics for human pathogens.

Intellectual property covering the use of lipopolysaccharide components to produce species and genus-specific antibodies against gram-negative bacteria, and to produce vaccines, is covered by Australian Petty Patent No. 660268 "Bacteria-Specific Lipopolysaccharide-Like Molecules". This work was done as a post-graduate project, which examined antigenic sub-fractions on the surface of gram-negative bacteria. Any future work utilising this intellectual property requires approval.

Further Developments

There were pathogenic gram-negative bacteria to which identifying mAbs were not made (e.g. *Edwardsiella ictalura*), and it is expected that as new bacterial pathogens of fish and shellfish arise, there will be a need to identify them. The techniques applied during this project could be utilised to produce antibodies against these bacteria.

Although the vaccine trials showed good protection against infection, via injections and oral routes, we were unable to produce a synthetic vaccine. The current method of vaccine production relies on extraction of bacterial cell-surface components, which is estimated to be of reasonably high cost. Therefore, no large-scale production has been carried out, but there are plans to chemically analysis the components and then

attempt produce synthetic vaccines. Due to their cross-protective properties, they would be of use to a wide range of veterinary and human situations (e.g. one that would convey protection to *Vibrio anguillarum* in salmonids, but also to *Vibrio cholerae* in humans).

Staff

Associate Professor Peter Hanna	Principal Investigator	1 Jul 93 -30 Jun 96	15%
Dr Desheng Chen	Research Associate	1 Jul 93 - 30 Jun 96	100%
Ms Marie McGlynn	Animal House Tech.	1 Jul 93 - 30 Jun 96	2%

Final Cost

	Allocated	Expenditure
FRDC Contribution	$169,761	$169,761
Deakin University Contribution	$170,500	$176,500
Contribution by Other Sources	$ 400	$ 400
TOTAL BUDGET	$340,661	$346,661

Distribution List of Final Report

This report will be distributed to State R&D Advisory Committees, researchers who have interests in diagnosing pathogens and fish vaccines, to several libraries, and to each of the following organisations.

Australian Fisheries Management Authority, Forrest, ACT

Australian Institute of Marine Science, Townsville, QLD

CSIRO Marine Laboratories, Division of Fisheries, Hobart, TAS

Division of Fisheries, CSIRO, Cleveland, QLD 4163

Mt Pleasant Laboratories, Department of Primary Industry & Fisheries, Launceston, TAS 7250

National Seafood Council, Deakin, ACT

NSW Fisheries, Brackish Water Fish Culture Research Station, Salamander Bay, NSW 2301

NSW Fisheries Research Institute, Cronulla, NSW

NSW Regional Veterinary Laboratory, Wollongbar, NSW 2477

Primary Industries (Fisheries) South Australia, Adelaide, SA

QDPI, Oonoomba Vet Lab, Townsville, QLD 4810

South Australian Fishing Industry Council, Port Lincoln, SA

South Australian Research and Development Institute (Aquatic Sciences), Henley Beach, SA

Tasmanian Fishing Industry Council, Sandy Bay, TAS

Tasmanian Sea Fisheries Division, Marine Research Laboratories, Taroona, TAS

Victorian Fisheries Branch, Department of Natural Resources and Environment, East Melbourne, VIC

Victorian Fishing Industry Federation, Melbourne, VIC

Western Australian Department of Fisheries, St. Georges Terrace, WA

Western Australian Marine Research Laboratories, Waterman, WA

Publications

Cartwright, G. A., Chen, D., Hanna, P. J., Gudkovs, N. and Tajima, K.(1994). Immunodiagnosis of virulent strains of *Aeromonas hydrophila* associated with epizootic ulcer syndrome (EUS) using a monoclonal antibody. J. Fish Dis., 17, 123-133.

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Appendix I

Development of Monoclonal Antibodies for the Identification of Gram-Negative Bacterial Pathogens

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Abstract

During the past few years we have developed an extensive library of monoclonal antibodies (mAbs) for the identification of fish, shellfish and human Gram-negative pathogens. All the mAbs are of murine background, in which whole cells or purified lipopolysaccharide (LPS) components of each Gram-negative strain were used to immunize BALB/c mice prior to performing fusions of spleen cells and Sp2/0-Ag-14 murine myeloma cells. Over 100 hybridomas have been cloned at limiting dilution, isotyped, screened for mAb production against whole cells of the immunizing strain, and then screened for specificity against a wide range of Gram-negative bacteria. Of these, about 30 that secrete mAbs with high affinity and putative potential for use in rapid diagnostic testing to identify bacteria, have been selected for commercial use. The mAbs identify strains *of Aeromonas, Edwardsiella, Escherichia, Flexibacter, Pseudomonas, Salmonella, Vibrio* and *Yersinia.* Some of these mAbs, particularly those against pathogenic *Vibrio* strains, have had extensive testing in research applications, and in practical situations identifying possible disease causing bacteria. One mAb has already been developed into a kit for the identification of *E. coli* O157 in contaminated foods and others are in the process of being developed into kits. MAbs against *Edwardsiella* strains are expected soon.

Introduction

In the late 1980's a project was implemented with an aim to develop monoclonal antibodies against Gram-negatives pathogens, starting with a number of *Vibrio* species that were pathogens of both fish, shell-fish and humans (Hanna *et al.*, 1992; Chen *et al.*, 1992). Overall, the need to have identifying monoclonal antibodies was threefold, and addressed: (1) disease situations, particularly of wild and cultured fish and shell-fish, but also including humans; (2) contaminated food products, as problems arise through human ingestion; and (3) contaminated waters, which can affect natural biosystems, aquaculture and human health.

Typical Gram-negative genera whose members include notable pathogens, include *Aeromonas, Edwardsiella, Escherichia, Flexibacter, Pseudomonas, Salmonella, Vibrio* and *Yersinia.* There has been a very large number of publications regarding these pathogens, and include such as books by Austin and Austin (1987) and Ellis (1988), as well as articles in several journals. It is not the purpose of this paper to present a review, but instead, describe the status of a library of mAbs developed for identifying Gram-negative pathogens in disease situations, monitoring contaminated environments such as food and water, and research purposes.

Materials and Methods

Bacteria and culture

Gram-negative bacteria used in the production of monoclonal antibodies (mAbs) are given in Table 1, and are listed in the order that immunizations of mice and subsequent cell fusions were performed. Initially, whole cells, that had been formalin-fixed or killed with sodium azide, were washed and used to immunize mice. In later work some isolated proteins (e.g. A protein) and cell-wall extracts (e.g. lipopolysaccharide; LPS) were used as immunogens. All bacteria were firstly cultured on plates using standard media (Bergey, 1984), after which single clones were used to inoculate appropriate liquid media for preparation of quantities up to 20 L. Following pelleting by centrifugation, bacteria were usually killed in 0.1% (w/v) sodium azide and then stored at 4°C until used, but some were killed in PBS containing 2% (v/v) formalin. In addition, cell-wall

LPS was either purchased from the Sigma Company or prepared by the hot phenol/water method (Westphal *et al.*, 1952).

Table 1. Bacterial strains used to prepare immunogens for monoclonal antibody production.

Fusion	Bacterial Strain[a]	Source[b]	Immunogen
F6	*Vibrio parahaemolyticus* WP1	HUH	Whole cells
F7	*Vibrio cholerae* Inaba 569B	HUH	Whole cells
F11	*Vibrio furnissii* ATCC 35016	JCUNQ	Whole cells
F12	*Vibrio harveyi* ACMM 130	JCUNQ	Whole cells
F13	*Vibrio anguillarum* AFHRL 1	AFHRL	Whole cells
F14	*Vibrio anguillarum* AFHRL 43	AFHRL	Whole cells
F15	*Vibrio alginolyticus* ACMM 101	JCUNQ	Whole cells
F16	*Vibrio damsela* ATCC 33537	JCUNQ	Whole cells
F17	*Aeromonas salmonicida* 85:9370-A	AFHRL	Whole cells
F18	*Vibrio ordalii* ATCC 33509	JCUNQ	Whole cells
F19	*Aeromonas salmonicida* 85:9370-A	AFHRL	A protein
F23	*Vibrio damsela* ATCC 33537	JCUNQ	Whole cells
F24	*Vibrio carchariae* ATCC 35084	JCUNQ	Whole cells
F25	*Vibrio* strains (F7, F14, F15 and F18)	Several	Mixed LPS components
F26	*Aeromonas hydrophila* 45	HU	Whole cells
F27	*Vibrio cholerae* 569B	Sigma	LPS component
F28	*Aeromonas hydrophila* 85:584-1A	AFHRL	LPS component
F29	*Aeromonas salmonicida* 85:9370-A	AFHRL	LPS component
F31	*Vibrio vulnificus* ACMM 106	JCUNQ	Whole cells
F32	*Vibrio anguillarum* AFHRL 02	AFHRL	Whole cells
F35	*Vibrio salmonicida* ACMM 677	JCUNQ	LPS component
F36	*Vibrio splendidus* ATCC 33125	JCUNQ	LPS component
F37	*Vibrio tubiashii* ATCC 19109	JCUNQ	LPS component
F38	*Escherichia coli* O157 EDL	RCH	Whole cells
F41	*Yersinia enterocolicita* IP 134	RCH	LPS component
F42	*Yersinia ruckeri* 85:8181-5A	FHU	LPS component
F43	*Salmonella typhimurium* L-2262	Sigma	LPS component
F44	*Vibrio mimicus* ATCC 33653	UQ	LPS component
F45	*Yersinia ruckeri* 88:8181-5A	FHU	LPS component
F46	*Flexibacter columnaris* 87:0023	FHU	LPS component

F47	*Flexibacter maritimus* 89:1763-5	FHU	LPS component
F48	*Burkholderia pseudomallei* NCTC 8016	UQ	LPS component
F49	*Pseudomonas aeruginosa* NCTC 10662	FHU	LPS component
F50	*Pseudomonas fluorescens* NCTC 10083	FHU	LPS component
F51	*Pseudomonas aeruginosa* NCTC 10662	FHU	LPS component
F52	*Escherichia coli* O111 Stoke W	RCH	Whole cells
F53	*Escherichia coli* O113 Batey C	RCH	Whole cells
F55[c]	*Edwardsiella ictaluri*	ATCC	LPS component

a ACMM, Australian Collection of Marine Microorganisms; ATCC, American Type Culture Collection.

b AFHRL, Australian Fish Health Reference Laboratory (now located at the Australian Animal Health Laboratories, CSIRO), Geelong; FHU, Fish Health Unit, Launceston; Dept. of Fisheries, HU, Hokkaido University, Hokodate, Japan; JCUNQ, James Cook University of North Queensland, Sir George Fisher Centre for Tropical Marine Studies, Townsville; RCH, Royal Children's Hospital, Melbourne; UQ, University of Queensland, Department of Microbiology, Brisbane.

c In preparation.

Production of monoclonal antibodies (mAbs)

The method described by Chen and Hanna (1992) has been routinely used to produce mAbs. It involved the *i.p.* immunization of BALB/c mice with washed whole bacterial cells, killed by sodium azide or formalin, or purified LPS components isolated from each Gram-negative strain. When antiserum titres were ≥1,000 the splenocytes were isolated and fused with the non-immunoglobulin secreting and HGPRT-deficient murine myeloma cells, known as Sp2/0-Ag-14 cells (Schulman *et al.*, 1978). Cell fusions were performed with a freshly prepared sterile solution of 45% (w/v) polyethylene glycol 4000 (Merck) and 5% (v/v) dimethyl sulphoxide (Merck) in distilled water (St. Groth and Scheidegger, 1980). Cells were normally diluted into seven 96-well culture plates, and hybridomas selected from other cells by growth in selective HAT medium (Littlefield, 1964). Monoclonality of useful hybridomas was achieved by sub-cloning hybridomas 2-3 times using a limiting dilution method (Goding, 1980), and mAbs isotyped using Bio-Rad isotyping kits.

Hybridomas have been cultured in RPMI 1640 medium (Cytosystems), supplemented with 10% (v/v) foetal calf serum (Cytosystems), 1mM glutamine (Cytosystems), 50 IU/mL penicillin (Cytosystems) and 50cmg/mL streptomycin (Cytosystems). All cultures have been grown at 37°C in a humidified air, containing 5% CO_2.

Screening using ELISA

Initial screening of hybridomas, secreting mAbs into supernatants, was carried out against whole cells of the bacterial strain used in the production of the original immunogen. This screening normally began at about 12 days following a fusion, and utilized an alkaline phosphatase ELISA in which positive supernatants produced a yellow colour in wells. Normally, negative controls had optical densities of less than 0.1 when read at A_{405} nm. In selecting hybridomas for use in diagnostic testing, we opted to choose those that produced optical densities of >1.0, and preferably those that were >3.0, following subtraction of controls. At this level any subtle variations between the results of differences strains were not important for identification purposes.

The ELISA conditions have been described previously (Chen and Hanna, 1992), and basically involved overnight coating of plates with washed sodium-azide killed bacterial cells suspended in a coating buffer, blocking of wells using 1% (w/v) bovine serum albumin, 3 washes with a 0.9% NaCl solution containing 0.05% Tween 20, incubation with hybridoma supernatants, another 3 washes with washing solution, incubation with goat-anti mouse Ig alkaline phosphatase conjugate (Silenus) diluted 1:1,000, a further 3 washes with washing solution, and a final enzymatic step involving an incubation with the substrate *p*-nitrophenyl-phosphate (Sigma). Positive clones were transferred to 24-well plates to multiply and produce larger quantities on mAbs, and were then recloned 2-3 times, rescreened, isotyped and frozen for long-term storage.

Cross-screening to determine mAb specificity also used an alkaline phosphatase ELISA. In this component of the research, to determine mAbs that identified only one specific bacterial group, the supernatants of 24 well-plates were utilised in duplicate ELISA that included a wide range of Gram-negative bacteria. The ELISA protocols were similar to that used in screening, and included positive and negative controls, using mAbs in supernatant or ascites, at dilutions of 10 to 1000. This range of mAb concentrations detected all bacteria. All screening assays were based on no cross-reactions occurring with other bacterial groups, and are therefore quite specific for the target strain. In

addition, the mAbs selected for diagnostic purposes have been isolated on their initial high reactivities and only subtle differences in reactivities with the same bacterial strains have been observed. As for most immunological based tests, only long-term and extensive use of the mAbs will show exceptions to their current diagnostic reliabilities.

Indirect immunofluorescence for rapid detection of Gram-negative pathogens

Detection of a particular Gram-negative strains by indirect immunofluorescence, using mAbs (see Table 2), relied primarily on an initial specific reaction of a mAb with an antigen on the surface of a bacteria. After a second reaction utilizing commercially available anti-mouse antibodies conjugated with fluorescein isothiocyanate (FITC; Silenus), the presence or absence of a mAb was visualised under a fluorescent microscope using narrow-band blue light. This technique has been applied to the rapid identification of cultured bacteria and bacteria in water samples (pelleted via centrifugation), and has also been used to identify bacteria attached to tissues on cryostat sections and smears of infected animal tissues (Chen and Hanna, 1992, 1994). At the dilutions of mAbs used in the tests, bacterial cells reacted equally strong in positive identifications and no non-specific reactions were observed with the different tissues.

Initial rapid tests typically involved using bacteria pelleted from water samples, and smears of tissues. Bacteria were also grown on nutrient agar plates overnight and a single representative colony aeseptically transferred and mixed in 1mL of PBS at pH 7.4, prior to testing. In all tests, aliquots of 20µL were smeared onto separate wells of teflon-coated microscope slides and after air-drying, the bacteria fixed to the glass by gentle heat over a burner. The primary reaction was carried out by carefully covering the dried smears with 20µL of each mAb (i.e neat supernatant or reconstituted ascites fluid, diluted 1:100 in PBS), and then placing the slides in damp incubation boxes for 45 min at 37°C. Positive and negative controls were included. The slides were washed at least 3 times with PBS, at pH 7.4, and left damp whilst 20µL of FITC-sheep anti-mouse Ig conjugate (Silenus), diluted 1:40 in PBS, was added to smears in each well. Following an incubation with the secondary conjugate for 45min at 37°C in the damp box, the slides were washed at least 3 times with PBS, at pH 7.4, and then mounted in 0.1mL of mounting solution (50mM PBS, pH 7.4, containing v/v 90% glycerol and 4%n-propyl gallate). Fluorescence of each smear was ranked as being none (-), weak (+), strong (++) or very strong (+++), depending on the relative fluorescence of bacteria. However, in most cases in which Gram-negative bacteria have been implicated in disease or water

monitoring situations, there has been a very strong positive identification of strains involved.

Results and Discussion

An extensive number of hybridomas have been produced against Gram-negative bacteria, and frozen for retention in long-term storage. Of these hybridomas, over 100 produced high affinity mAbs with potential for use in rapid diagnostic testing. From this group, over 30 have been selected for commercial development, and the mAbs identify strains *of Aeromonas, Escherichia, Flexibacter, Pseudomonas, Salmonella, Vibrio* and *Yersinia* (Table 2). Some of these mAbs, particularly those against pathogenic *Vibrio* strains, have had extensive testing in research applications, and in practical situations identifying possible disease-causing bacteria (unpublished data). Some have had limited testing, and will be replaced by reserves if they show, as yet, undetected problems. An ELISA kit for the rapid identification of *E. coli* O157 in contaminated foods has been developed from one of several mAbs against the O157 serotype. Others mAbs are in the process of being developed into kits, and this will be extended as demand prevails. Meanwhile, mAbs against the *Edwardsiella* strains, *E. tarda* and *E. ictaluri* are currently being developed.

Table 2. Monoclonal Antibodies with Diagnostic Capabilities

Monoclonal Antibody[a]	Specificity (Genus/Species)	Isotype	Heat stability[b] (LPS/Protein)	Comments[c]
F29P5B8	*Aeromonas*	IgM/κ	S (LPS)	
F29P3C2	*A. salmonicida*	IgM/κ	S (LPS)	
F19P5E12	*A. salmonicida*	IgG_1/λ	L (Protein)	A protein
F26P5C8	*A. hydrophila*	IgG_3/λ	S (LPS)	
F38P2E12	*Escherichia*	IgG_{2b}/κ	S (LPS)	being tested
F38P6G9	*E. coli* O157	IgG_1/κ	S (LPS)	kit form only
F47P4D12	*Flexibacter*	IgM/κ	S (LPS)	
F46P6A2	*F. columnaris*	UD	UD	weak

F47P7A3	*F. maritimus*	IgM/κ	S (LPS)	
F43P5C12	*Salmonella*	IgA/λ	S (LPS)	being tested
F41P4F9	*Yersinia*	IgA/κ	S (LPS)	
F41P2C1	*Y. entocolictica*	IgA/λ	S (LPS)	weak
F45P4D5	*Y. ruckeri*	IgG_{2b}/λ	S (LPS)	weak
F11P4F11	*Vibrio*	IgG_1/κ	S (LPS)	excellent for *Vibrio*
F13P1F3	*V. anguillarum*	IgG_1/κ	S (LPS)	
F15P1A7	*V. alginolyticus*	IgG_{2a}/κ	L (Protein)	
F24P5G6	*V. carchariae*	IgG_3/κ	PS (LPS)	
F7P5C11	*V. cholerae*	IgG_{2b}/κ	S (LPS)	
F7P2A5	*V. cholerae*	IgG_3/κ	S (LPS)	O1 strains
F27P5H10	*V. cholerae*	IgM/λ	S (LPS)	non O1 strains
F12P4E11	*V. harveyi*	IgG_1/κ	PS (LPS)	
F44P2E3	*V. mimicus*	IgG_3/κ	S (LPS)	
F18P6C6	*V. ordalii*	IgG_3/κ	S (LPS)	
F6P5C5	*V. parahaemolyticus*	IgA/κ	S (LPS)	
F35P3F12	*V. salmonicida*	IgM/κ	S (LPS)	
F36P3B10	*V. splendidus*	IgM/κ	S (LPS)	
F37P2B7	*V. tubiashii*	IgA/λ	S (LPS)	weak
F31P5B8	*V. vulnificus*	IgG_2/κ	S (LPS)	
F23P7E7	*V. damsela*	IgM/κ	PS (LPS)	not *Vibrio* with F11P4F11
F48P1B1	*B. pseudomallei*	IgM/κ	S (LPS)	was *P. pseudomallei*
F51P4C4	*Pseudomonas*	IgG_3/κ	S (LPS)	
F50P1B5	*P. fluorescens*	IgA/λ	S (LPS)	weak
F51P1F2	*P. aeruginosa*	IgA/λ	S (LPS)	

a Available from TECRA Diagnostics, 28 Barcoo St., Roseville, NSW 2069, Australia.

b S, stable; L, labile.

c All with strong-very strong reactions, unless stated otherwise.

UD Undetermined

The scope of applications using the Gram-negative mAbs has been increasing. For example, they have been employed in the rapid identification of bacteria in infected tissues, cultured bacteria, bacteria in water samples, following pelletisation via centrifugation, bacteria in food for human consumption, and human clinical situations. Some have also been used in research situations to study the differential attachment of Gram-negatives to cryostat sections of tissues, and smears of infected animal tissues (Chen and Hanna, 1992, 1994). Another was developed and used in the immunodiagnosis of virulent strains of *Aeromonas hydrophila* associated with epizootic ulcer syndrome (Cartwright *et al.*, 1994). A project drawing considerable interest is an investigation into the potential use of conjugating different fluorochromes onto genus-specific Gram-negative mAbs for use in rapid monitoring of water quality.

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