IMMUNE RESPONSE OF BARRAMUNDI (*Lates calcarifer*) TO *Vibrio harveyi* BACTERIN

by

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ABSTRACT

Commercial farming of barramundi or Asian seabass (*Lates calcarifer*) is a burgeoning industry in Australia and South East Asia. At present there are few serious bacterial infections, however disease caused by *Vibrio harveyi* infection is an on-going problem with the potential to become chronic as the industry expands. Therefore, the development of an effective vaccine against vibriosis was seen as a prudent strategy for the future success of barramundi production. This thesis reports on the development of appropriate reagents for *in vitro* monitoring of immune responses to a bacterin, and subsequent validation in laboratory trials where fish were immunised with a bacterin via various routes or, with lipopolysaccharide (LPS) via intraperitoneal (IP) injection.

Barramundi immunoglobulin was isolated by affinity chromatography using either mannan-binding protein (MBP) or Staphylococcal protein A (SpA) as capture ligands. Both isolation products were used to produce polyclonal antisera in rabbits, which were subsequently compared for specificity and titer. Two antisera were used to monitor anti-*V. harveyi* antibody activity in barramundi serum after primary and secondary immunisations with bacterin via the IP route. Elevated antibody levels after secondary immunisation indicated a memory response.

The routes of bacterin administration were investigated in terms of antibody production and some non-specific immune parameters. It was found that barramundi respond systemically to bacterin delivered by IP injection, immersion (IMM) and anal intubation (AI). The highest and most consistent response was in fish treated via IP followed by AI then IMM. This consistency of response from IP-treated fish carried over to bacterial inhibitory activity of serum where all demonstrated >50% inhibitory activity relative to non-immune controls. There was no significant enhancement of serum lysozyme activity nor head kidney macrophage phagocytic activity as a result of any immunisation procedure.

Finally, the response of barramundi to IP immunisation with bacterin or lipopolysaccharide (LPS) extracted from the bacterium was compared to elucidate the role of the latter. There

were systemic antibody responses to both preparations with little difference noted in the magnitude of the response. However, bacterial inhibitory activity of serum was highest in the bacterin treament group. This may indicate that anti-LPS antibodies do not agglutinate to the same extent as anti-whole bacterial cell antibodies.

In conclusion, systemic antibody and bacterial inhibitory activity after immunisations with the bacterin suggests that it may be an effective vaccine and oral administration is worth investigation. The protective role of LPS is unclear, though relatively lower bacterial inhibitory activity of anti-LPS antibodies suggests it may be less protective than anti-whole bacterial cell antibodies.

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LIST OF ABBREVIATIONS

AI	anal intubation
ANOVA	analysis of variance
ASC	antibody-secreting cells
C	complement
Con A	concanavalin A
DAB	3,3' diaminobenzidine tetrachloride peroxidase
ELISA	enzyme-linked immunosorbent assay
FCS	forward scatter
FPS	fish physiological saline
GALT	gut-associated lymphoid tissue
HC	heavy chain mmunoglobulin
HC 1	rabbit anti-barramundi heavy chain immunoglobulin 1
HC 2	rabbit anti-barramundi heavy chain immunoglobulin 2
HGG	human gamma globulin
HRP	horseradish peroxidase
Ig	immunoglobulin
IMM	immersion
IP	intraperitoneal
IS	immune serum
LC	light chain immunoglobulin
LC 1	rabbit anti-barramundi light chain immunoglobulin 1
LC 2	rabbit anti-barramundi light chain immunoglobulin 1
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
MBP	mannan-binding protein
MBP 3	rabbit anti-barramundi MBP-derived immunoglobulin 3
MBP 4	rabbit anti-barramundi MBP-derived immunoglobulin 4
MTT	3-(4,5-dimethylyhiozol 1-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight

NB 2	nutrient broth no. 2
NIS	non-immune serum
OD	optical density
OPD	*o*-phenylenediamine
PAGE	polyacrylamide gel electrophoresis
PBLs	peripheral blood leucocytes
PBS	phosphate buffered saline
PMSF	phenylmethyl-sulphanol fluoride
SDS	sodium dodecyl sulphate
SE	standard error
SpA	staphylococcal protein A
SSC	side scatter
TBS	tris buffered saline
Ve	elution volume
Vo	void volume

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CHAPTER 1

GENERAL INTRODUCTION

As aquaculture expands and attempts to make up the worldwide deficit between supply from wild fisheries and demand for fish the risk of disease in these ventures also grows. The intensive nature of aquaculture imposes stresses which are regarded as being a significant contributor to the susceptibility of fish to diseases (Stoskopf, 1993). In addition, the high stocking densities employed allow for a greater risk of infectious contact between individuals and thereby precipitating epizootics. Good animal husbandry together with the use of disinfectants and therapeutants such as antibiotics have done much to lessen disease impact. However, they are no longer sufficient to adequately control disease problems in aquaculture. Moreover, the use of antibiotics and other chemicals has led to concerns over drug resistance and environmental contamination and may lead to restricted use of some chemicals. In any case therapeutants such as antibiotics are expensive, may require multiple doses and are generally applied only after fish show signs of disease (Horne and Ellis, 1988). Therefore, it is prudent to continue investigations into prophylactic measures such as vaccination as a means of controlling disease.

The exhibition of adaptive immunity by fish suggested that vaccination would be a feasible method of disease control. Although immunisation of fish was attempted in the 1940s (Duff, 1942) the economic impetus to protect cultured fish stocks did not arise until the 1970s (Newman, 1993). Since then there has been much success with vaccines against bacterial pathogens. Fundamentally, the development of effective vaccines requires understanding of the immune system together with the nature of the antigens which may induce a protective response on exposure to fish. To that end much of the research in the field of fish immunology has focussed on defining and understanding fish immune systems in comparison to those of mammals. These tasks have been made easier by employing genetic and biochemical techniques which enable minute examination of the machinery and

processes involved. Thus, during the 1980s and 1990s, the research has broadly gone in two directions: furthering an understanding of fish immunity and, application of this knowledge to enhance protection from disease in cultured fish. Aside from knowledge useful in a practical sense, the first direction provides, from a holistic perspective, fundamental data on the evolution of immune systems (Miller *et al.*, 1998). The second encompasses characterisation of antigenic components of pathogens, ontogeny of immune system of fish, vaccine administration routes and formulation and trials of prototype vaccines. The magnitude of research performed and reported and the diversity of the species studied are virtual acknowledgement of fish vaccinology as a discipline in its own right (Ellis, 1999a).

1.1 IMMUNITY IN TELEOST FISH

The immune system of teleost fish is fundamentally similar to that of higher vertebrates and, therefore, fish are generally well-equipped to protect themselves against pathogenic organisms. Teleosts possess both humoral and cell-mediated immunity which are further classified as specific and non-specific. Vaccination allows a host to develop resistance against a disease by stimulating specific and non-specific immunity and considerable interaction between both immune arms occurs in an effective response. Numerous recent reviews and texts have more than adequately dealt with aspects of piscine immune mechanisms (e.g. Sakai, 1992; Kaattari, 1994; van Muiswinkel, 1995; Iwama and Nakanishi, 1996; Miller *et al.*, 1998; Partula, 1999), therefore this introduction will concentrate more on the overview and the pertinence of work in the field of fish vaccinology.

Vaccination can activate acquired immunity in a host to specific antigens and such immunity is characterised by specificity and memory (Warr, 1996). Fish display cellular and humoral immunity which have these characteristics (van Muiswinkel, 1995) and are therefore amenable to vaccination. Many of the existing immune components in fish are similar to those in mammals, for example equivalents of T and B lymphocytes which are the effector cells of acquired immunity and antigen-presenting or accessory cells which include

monocyte/macrophages (Vallejo *et al.*, 1992.). The B cells, with surface immunoglobulin, (sIg^+) can be distinguished from lymphoid cells with no surface Ig (sIg^- i.e. T cells) by monoclonal antibodies (mAbs) (Miller *et al.*, 1998). T cells can also be distinguished using mAbs to T cell specific markers in fish species such as carp, *Cyprinus carpio*, (Rombout *et al.*, 1997; Rombout *et al.*, 1998) European seabass, *Dicentrarchus labrax*, (Scapigliati *et al.*, 1995) and channel catfish, *Ictalurus punctatus* (Passer *et al.*, 1996). In terms of mitogenic responses, it has been demonstrated that channel catfish have T helper cells, B cells and accessory cells similar to those of mammals (Sizemore *et al.*, 1984; Miller *et al.*, 1985). This was shown using known T and B cell mitogens such as concanavalin A (Con A) and lipopolysaccharide (LPS) respectively. Observations of sIg^+ response to LPS but not Con A suggested sIg^+ of catfish to be B cells and sIg^- cells were thought to be T cells based on mitogenic responses to Con A, these cells also responded to LPS which is in contrast to mammalian T cells (Sizemore *et al.*, 1984). Similarly in catfish, an *in vitro* antibody response to a thymus-dependent antigen in a sIg^+ lymphocyte population required the presence of sIg^- lymphocytes and accessory cells whereas when a thymus-independent antigen was used only sIg^+ lymphocytes and accessory cells were required (Miller *et al.*, 1985).

Notwithstanding the similarities, piscine immunity is often seen as simplistic compared to immunity in higher vertebrates. Aside from the phylogenetic position of fish relative to mammals, reasons for this perception are, in part, due to comparisons being made between two systems where only one has been extensively studied (Kaattari, 1998). Another reason relates to current knowledge of the differences between the acquired immune responses of teleosts and mammals. Immunological memory in fish is less complex than that in higher vertebrates (Newman, 1993), for example fish are thought not to possess physiologically distinct memory cells and an elevated secondary humoral response may be due to an increase in the antigen-specific B cell pool (Arkoosh and Kaattari, 1991). This is important as the concept of immunological memory is one basis for vaccination. The predominant class of teleost Ig is thought to be equivalent to mammalian IgM (Pilström and Bengtén, 1996). Even though isotypic variation is accepted in Ig of fish (Kaattari, 1994) isotype switching within a single B cell is yet to be conclusively demonstrated. Similarly, affinity

maturation, manifest as a logarithmic increase in antibody affinity, has not been observed (Arkoosh and Kaattari, 1991). Kaattari (1994) postulated that reasons for a lack of affinity maturation may be due to nature of the response. For example, T suppressor cells seem to exert a more intense control on antibody affinity than in mammals as evidenced in studies where T suppressor cells were removed via gentle radiation and subsequent increase in antibody activity observed (Kaattari *et al.*, 1986). A different theory for lack of affinity maturation in teleosts concerns the way in which it is assessed (Kaattari 1994). Commonly, measurement of affinity maturation is determined from individual binding sites on an Ig molecule however, as fish predominantly have tetrameric IgM then modest affinity increases at each binding site may be magnified logarithmically when the functional affinity of the whole molecule is assessed (Kaattari, 1994). This may indicate that fish immunity has evolved differently to that of mammals and that using mammalian criteria to define fish immunity may lead to erroneous interpretation (Kaattari, 1994). The tools required to properly elucidate fish immunity, such as monoclonal mAbs to T cell markers are currently limited (Rombout *et al.*, 1998). However, at present this is a very active area of research as it has profound implications in discerning the ontogeny of T cells and subsequent development of an active inducible immune response.

A consistent theme in some research into fish immune systems through the 1980s and 1990s was identification and elucidation of mucosal immunity. The importance to fish of this apparently autonomous immune compartment is highlighted by the fact the skin provides another mucosal barrier in addition to the gill and alimentary tract. A degree of autonomy of the mucosal immune system is indicated by observations in mammals that mucosal and systemic immunity can be stimulated if immunisation occurs by a mucosal route, however systemic immunisation does not usually induce mucosal immunity (McGhee *et al.*, 1992; Hathaway and Kraehenbuhl, 2000). After antigen delivery to lymphoid tissue in mucosal inductive sites (i.e. gastrointestinal, respiratory and genitourinary tracts) and presentation to lymphocytes, stimulated T and B cells are transported via lymphatic, then systemic, circulation to distant mucosal sites where B cells are selectively retained (McGhee *et al.*, 1992). Clarification of this common mucosal immune system will aid in vaccine design and delivery to the mucosal sites with the aim to induce protection at the site of infection

(McGhee *et al.*, 1992). This is equally important in fish as most horizontally transmitted pathogens enter fish via the mucosal barriers (Evelyn, 1996). In examining mucosal immunity in fish the research initially looked at secretory Ig and antibody activity was described in fish mucus during the 1970s (e.g. DiConza and Halliday, 1971). Some examples of descriptions and elicitation of Ig in fish appear in Table 1.1. The elicitation of antibody in skin mucus with little or no concomitant serum antibody after oral antigen delivery (Fletcher and White, 1973; Kawai *et al.*, 1981; Rombout *et al.*, 1993a) indicated a degree of autonomy of mucosal immunity. Further, Lobb (1987) elicited antigen-specific antibody in the mucus of channel catfish after immersion in dinitrophenylated horse serum albumin but found no increase in post-immersion antibody activity in the serum. Although Wong *et al.* (1992) found significant mucus and serum antibody levels in coho salmon after oral administration of an enterically coated *V. anguillarum* vaccine, Cain *et al.* (2000) noted an important difference. These authors found antibody in the mucus and serum of rainbow trout using a hapten-carrier antigen delivered by IP injection and anal intubation but noted that peak mucus response occurred 2 weeks prior to the peak serum response. This may indicate independent antibody production within mucosal and systemic compartments (Cain *et al.*, 2000).

Lobb and Clem (1981) suggested that Ig present in cutaneous mucus and bile of channel catfish was locally synthesised after demonstrating that Ig was not transudated nor actively transported from the serum. Rombout *et al.* (1993a) demonstrated antigenic differences between the heavy chains of mucus and serum Ig in carp using mAbs. In fish the mucosal inductive sites are those where lymphoid cells, but not distinct tissue, have been shown to be present. Such sites include the intestine (Rombout *et al.*, 1986) and to a lesser extent leucocytes are also present in the skin and gills (van Muiswinkel, 1995). The mechanisms required for antigen uptake and transport across the epithelium to these cells are all present in the intestine (Rombout *et al.*, 1993b) and apparently the skin and gills. Moore *et al.* (1998) quantified particulate antigen in various tissues of rainbow trout after immersion in a suspension of polystyrene microspheres linked to bovine serum albumin (BSA). These authors observed persistence of the particles for up to 24 days in epithelial cells of the skin and phagocytes underlying the epithelia of both the skin and gills and relatively small

numbers of particles in the kidney and spleen. It was suggested that retention of antigen at the site of uptake was consistent with the role of local immune responses and protection after immersion immunisation with little or no concomitant systemic antibody production (Moore *et al.*, 1998). Further evidence of a distinction in mucosal and systemic immunity is the apparent difference in the kinetics of antibody-secreting cells (ASC) in both compartments (Kaattari and Piganelli, 1996). Davidson *et al.* (1993) found ASC in both the head kidney and intestinal mucosa of rainbow trout after intraperitoneal (IP) injection and oral intubation with an *Aeromonas salmonicida* bacterin, although differences were noted in each depending on the immunisation route. Antigen-specific ASC appeared in the head kidney 2 weeks after IP injection but were not noted until 7 weeks later in the intestinal mucosa (Davidson *et al.* 1993). Conversely, ASC appeared simultaneously in both tissues after oral intubation with the bacterin (Davidson *et al.* 1993).

Much of the evidence suggests that, as in mammals, fish possess a mucosal immune system that can, to some extent, act independently of systemic immunity and work continues in this area. Therefore, as in mammalian vaccinology, full elucidation of the mucosal immune system will mean that researchers will be in a much better position to devise strategies that optimise enhancement of immunity.

Table 1.1 Examples of existence and detection of mucosal immunoglobulin in fish

Species	Mucus source	Antigen	Administration route	Detection method
Catfish (*Tachysurus australis*)	Skin	*Salmonella enteritis* bacterin	Intraperitoneal (IP) injection	Bacterial agglutination [1]
Gar (*Lepisosteus platyrhincus*)	Skin	Sheep red blood cells	IP injection	Haemagglutination [2]
Plaice (*P. platessa*)	Skin	*Vibrio anguillarum* bacterin	IP injection	Haemagglutination [3]
Rainbow trout (*Oncorhynchus mykiss*)	Skin	*V. anguillarum* bacterin	IP injection	Bacterial agglutination [4]
Channel catfish (*Ictalurus punctatus*)	Skin	*Salmonella paratyphi* bacterin	IP injection	Bacterial agglutination [5]

Table 1.1 Continued

Species	Mucus source	Antigen	Administration route	Detection method
Rainbow trout (*O. mykiss*)	Skin	Sheep red blood cells	IP injection	Haemagglutination [6]
Tilapia (*Oreochromis mossambicus*)	Skin	Human gamma globulin (HGG)	IP injection	Enzyme-linked immunosorbent assay (ELISA) [7]
Channel catfish (*I. punctatus*)	Skin/Intestine	*Edwardsiella ictaluri* bacterin/outer membrane proteins	IP injection/Anal	ELISA [8]
Channel catfish (*I. punctatus*)	Skin	Dinitrophenylated horse serum albumin	Immersion	Haemagglutination [9]
Plaice (*P. platessa*)	Skin	*V. anguillarum* bacterin		Haemagglutination [3]

Table 1.1 Continued

Species	Mucus source	Antigen	Administration route	Detection method
Ayu (*Plecoglossus altivelis*)	Skin	*V. anguillarum* bacterin	Oral	Bacterial agglutination [10]
Carp (*Cyprinus carpio*)	Skin	Soluble ferritin	Oral	ELISA [11]
Coho salmon (*Oncorhynchus kisutch*)	Skin	*V .anguillarum* lyophilised and enteric coated	Oral	ELISA [12]
Tilapia (*O. mossambicus*)	Skin	HGG in micelles	Oral/Anal	ELISA [7]
Carp (*C. carpio*)	Intestine	*V. anguillarum* bacterin	Anal	ELISA [13]

Table 1.1 Continued

Species	Mucus source	Antigen	Administration route	Detection method
Rainbow trout (*O. mykiss*)	Gill	*Flavobacterium branchiophilum* bacterin	IP injection/Immersion	ELISA [14]
Turbot (*Scophthalmus maximus*)	Skin/Intestine	LPS from *Cytophaga*-like bacterium LPS coated latex	IP injection Oral/Immersion	Haemagglutination [15]
Rainbow trout (*O. mykiss*)	Skin	Fluorescein isothiocyanate conjugated to keyhole limpet haemocyanin	IP injection/Anal	ELISA [16]
Rainbow trout (*O. mykiss*)		Encapsulated HGG	Oral	ELISA [17]

[1] DiConza and Halliday, 1971

[2] Bradshaw *et al.*, 1971

[3] Fletcher and White, 1973

[4] Harrel *et al.*, 1976

[5] Ourth, 1980

[6] St. Louis-Cormier *et al.*, 1984

[7] Jenkins *et al.*, 1994

[8] Ainsworth *et al.*, 1995

[9] Lobb, 1987

[10] Kawai *et al.*, 1981

[11] Rombout *et al.*, 1989

[12] Wong et al., 1992

[13] Rombout *et al.*, 1986

[14] Lumsden *et al.*, 1995

[15] Al-Harbi and Austin, 1992b

[16] Cain *et al.*, 2000

[17] Lavelle *et al.*, 1997

1.2 VACCINATION AGAINST BACTERIAL PATHOGENS

The simple approach of inactivating a culture of a pathogen and administering it to a potential host has been an effective vaccine strategy in many situations. Primary examples include the first vaccines developed for vibriosis and yersiniosis/enteric redmouth caused by *Vibrio anguillarum* and *Yersinia ruckeri* infections respectively. Mass immunisation via immersion is highly effective for yersiniosis (Ellis, 1988a). Similarly, initial vaccines developed against *V. anguillarum* also displayed good protection (e.g. Antipa and Amend, 1977; Gould *et al.,* 1978) and paved the way for subsequent use of inactivated cultures as vaccines. In general these inactivated cultures or bacterins comprise a crude mixture of whole cells, cell debris and intracelluar and extracellular products some of which are immunogenic and provide protection. In fact most of the currently available commercial vaccines against bacterial pathogens are bacterins or their components albeit in some cases delivered with adjuvants (Newman, 1993). This vaccination strategy has not worked in all cases, a striking example was the failure for a number of years to produce a consistently effective vaccine against furunculosis, a disease caused by infection with *Aeromonas salmonicida*. The complex relationship between host and pathogen, expression of different substances when residing in the host or a culture medium and, the ability of the organism to circumvent some host defence mechanisms all conspired to make vaccine development difficult. Such an example underlines the need for a more systematic approach to vaccine design where immunogenic determinants are identified in particular those associated with virulence. These investigations also have the potential to uncover determinants that may provide cross-protection within strains of a pathogen, for example it is desirable for an effective furunculosis vaccine to confer protection against disease caused by atypical *A. salmonicida* strains (Hastings, 1988).

Different approaches are also required when confronted with a pathogen such as *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonids. This organism is both an extracellular and intracellular pathogen, residing in monocytes and macrophages (Bruno, 1986) where it auto-agglutinates and is possibly protected against

enzymatic degradation (Bruno, 1988). *R. salmoninarum* is fastidious in its growth requirements and extremely long generation times make isolation from tissues and then culture very time consuming (Newman, 1993). These characteristics pose problems with methods used for testing efficacy of vaccines. The rapid development of molecular biological techniques aiding in antigen identification and subsequent production of subunit and DNA vaccines will help overcome some of the problems associated with vaccine development for the more troublesome pathogens. There are now several commercially available vaccines used against bacterial pathogens in finfish culture throughout the world and these together with the vaccine development status of other bacterial pathogens are detailed in Table 1.2.

The types of vaccines used and under development against bacterial pathogens include bacterins, incorporating monovalent and polyvalent preparations, and live attenuated organisms. The use of polyvalent vaccines seem to have two advantages: protection can be conferred against multiple pathogens and there are sometimes synergistic effects. For example, commercial vaccines are available that are made up of *V. anguillarum*, *V. salmonicida* and *A. salmonicida* antigens with adjuvant which are routinely used in Norway (Press and Lillehaug, 1995; Hoel *et al.*, 1998). A synergistic effect with this polyvalent vaccine was noted in experiments where enhancement of protection against furunculosis was found relative to groups given *A. salmonicida* antigens only (Midtlyng *et al.*, 1996). Hoel *et al.* (1997) subsequently proposed that the *V. salmonicida* component was probably responsible for the enhancement and effectively acted as an adjuvant. Concerns about antigenic competition in polyvalent vaccines and possible suppressive effects were partially dispelled when Amend and Johnson (1984) found no antigenic competition in salmonids when administered various combinations of *V. anguillarum, Y. ruckeri, A. salmonicida* and *R. salmoninarum* bacterins.

The potential of live vaccines for cultured fish has been recognised and appears to offer distinct benefits. Live vaccines can activate responses similar to those seen during natural infection, particularly cell-mediated responses (Marsden *et al.*, 1996a) required for elimination of intracellular pathogens (Kaufmann, 1995) and, they may express antigenic

substances *in vivo* that may be lacking during *in vitro* culture as in bacterin manufacture (Brown *et al.*, 1993). Another advantage of vaccination with live attenuated bacteria is that it is effectively an infection and therefore relatively lower doses could be used to initiate the infection and then, if bacterial cells are shed, could facilitate dissemination throughout a population (Gudding *et al.*, 1999). Genetically attenuated strains of *A. salmonicida* have been proposed as vaccines against furunculosis (Vaughan *et al.*, 1993; Thornton *et al.*, 1994; Marsden *et al.*, 1996a; Marsden *et al.*, 1996b) to exploit these advantages, and experimental results have been promising. The major disadvantages include the risk of a return to virulence, and adverse perceptions of using genetically modified organisms on fish farms. These factors are likely to impede commercial licensing.

Table 1.2 Bacterial diseases in finfish and current status regarding vaccine development

Disease-Species affected	Causative organism	Existing/proposed vaccine type	Administration method	Development status
Vibriosis-farmed marine fish	*Vibrio anguillarum*	Bacterin	Injection/Immersion	Commercially available [1,2]
Vibriosis-farmed marine fish	*Vibrio ordalii*	Bacterin	Injection/Immersion	Commercially available [1,2]
Cold water vibriosis Hitra disease-salmonids	*Vibrio salmonicida*	Bacterin	Injection/Immersion	Commercially available [1,2]
Winter ulcer disease-farmed Atlantic salmon	*Vibrio viscosus*	Polyvalent Bacterin with adjuvant	Injection	Not commercially available, experimental results good [2,3]

Table 1.2 Continued

Disease - species affected	Causative organism	Existing/proposed vaccine type	Administration method	Development status
Yersiniosis/Enteric red mouth-salmonids	*Yersinia ruckeri*	Bacterin	Immersion	Commercially available [1,2]
Furunculosis-salmonids	*Aeromonas salmonicida* subsp. *salmonicida*	Bacterin with adjuvant	Injection/Immersion	Commercially available,[1,2] research on-going
Ulcer disease-numerous farmed fish	*A. salmonicida* atypical strains	Bacterin	Injection	Commercially available, [2] research on-going [4]
Aeromonad septicemia-numerous farmed fish	*Aeromonas hydrophila*	Bacterin or attenuated live cells	Injection/Oral	Not commercially available, research on-going [5,6]

Table 1.2 Continued

Disease- Species affected	Causative organism	Existing/proposed vaccine type	Administration method	Development status
Pasteurellosis/ Pseudotuberculosis- farmed marine fish	*Photobacterium damsela* subsp. *piscida*	Bacterin or antigenic components	Injection/Immersion	Not commercially available, research on-going [2,7]
Edwardsiellosis- eels, marine fish	*Edwardsiella tarda*	Bacterin or outer membrane protein	Injection	Not commercially available, research on-going [8]
Enteric septicemia- channel catfish	*Edwardsiella ictaluri*	live attenuated cells	Immersion	Not commercially available, experimental results good, research on-going [9]
Bacterial kidney disease- salmonids	*Renibacterium salmoninarum*	Modified bacterin	Injection/Oral	Not commercially available, experimental results good, research on-going [10]
Cutaneous erosion, skin/tail rot- numerous farmed fish	*Flexibacter spp* *Cytophaga spp*	Bacterin (*F. columnaris*)	Injection	Not commercially available, research on-going [11]

Table 1.2 Continued

Disease-Species affected	Causative organism	Existing/proposed vaccine type	Administration method	Development status
Rickettsial septicemia-salmonids	*Piscirickettsia salmonis*	Bacterin	Injection	Not commercially available, research on-going [12,13]
Streptococcosis-numerous farmed fish	*Streptococcus iniae*	Bacterin	Injection	Not commercially available, experimental results good, research on-going [14,15]
Lactococcosis-rainbow trout, yellow tail	*Lactococcus garviae*	Bacterin	Injection	Not commercially available, research on-going [16]

1. Press and Lillehaug, 1995
2. Haenen, 2000
3. Gregor and Goodrich, 1999
4. Wiklund and Dalsgaard, 1998
5. Leung *et al.*, 1997
6. Azad *et al.*, 1999
7. dos Santos, 2000
8. Tu and Kawai, 1999
9. Wise *et al.*, 2000
10. Piganelli *et al.*,1999
11. Bader *et al.*, 1997
12. Smith *et al.*, 1995
13. Almendras and Fuentealba, 1997
14. Eldar *et al.*, 1997
15. Klesius *et al.*, 2000
16. Akhlaghi *et al.*, 1996

1.3 BARRAMUNDI (*Lates calcarifer*) AQUACULTURE

Lates calcarifer is a euryhaline species which is a member of the subfamily Latinae from the family Centropomidae (Nelson, 1994). This species is widely distributed throughout the Indo-West Pacific region (Grey, 1987) where it is variously known as seabass or Asian seabass or, in Australia and Papua New Guinea, barramundi (Rimmer and Russell, 1998). The fish is protandrous, catadromous species that is particularly suited to culture. It is extremely fast-growing and very tolerant of relatively poor environmental conditions. Moreover, in Australia, it has been traditionally a popular recreational species renowned for its angling and flesh qualities, the latter attribute ensuring a robust market for the farmed product. Chief producers of barramundi worldwide are Indonesia, Malaysia, Philippines and Thailand (Grey, 1987). In Australia, barramundi aquaculture is a burgeoning industry as evident by the growth in production from 219t in 1993 (FAO, 1995) to approximately 633t in 1997/98 (O'Sullivan and Roberts, 2000).

Barramudi are cultured in either freshwater, brackishwater or seawater, though their catadromous nature means that broodstock require seawater to facilitate final gonadal maturation (Rimmer and Russell, 1998). The larvae are also reared in seawater but at the juvenile stage they may be on-grown in brackishwater or freshwater.

In both nursery and grow-out phases cultured barramundi are prone to diseases from a range of pathogenic organisms. Although parasitic, fungal and viral infections do occur these are less common than bacterial diseases (Anderson and Norton, 1991). Parasites including ciliated protozoans, trematodes and crustaceans have all been reported (Choncheunchob *et al.*, 1987; Glazebrook and Campbell, 1987; Humphrey and Langdon, 1987) but do not appear to be major concerns. Periodic mass mortalities at the larval stage early in the development of the industry have variously been attributed to dietary deficiencies (Rodgers and Barlow, 1987) and to a viral encephalopathy caused by a picorna-like virus (Glazenbrook, 1990). The virus has since been shown to be antigenically similar to nodavirus (Munday *et al.*, 1994) and in fact was identified as belonging to the family

Nodaviridae (Comps *et al.*, 1994). However, the institution of improved hatchery techniques has been very successful in controlling outbreaks of this disease (Munday *et al.*, 1994). Most commonly, mortalities in farmed barramundi are caused by bacterial infections (Anderson and Norton, 1991) such as those by *Streptococcus iniae* (Bromage *et al.*, 1999). In addition, diseases caused by bacteria of the genera *Vibrio, Aeromonas, Pasteurella, Streptococcus* and *Flexibacter/Cytophaga* have all been reported (Rimmer and Russell, 1998). Vibriosis has been associated with poor environmental and nutritional conditions in some instances (Subasinghe and Shariff, 1992) suggesting that improved husbandry techniques may aid in control of this disease. In Australia, there is an on-going disease problem with vibriosis caused by infection with *Vibrio harveyi* (Rachel Webb, pers comm) which occurs during the seawater and brackishwater part of the culture period. Though there have been no serious losses due to this disease in Australia, the potential for vibriosis to become a chronic problem exists, particularly if the industry intensifies as the growth in production figures suggest. Therefore, the development of an effective vaccine against vibriosis caused by *V. harveyi* infection was seen as a prudent strategy for the future success of barramundi production, particularly since antibiotic resistant isolates have already been described in penaeid prawn culture (Karunasagar, 1994).

1.4 THE PATHOGEN *Vibrio harveyi*

Members of the Vibrio genus of bacteria have been described as the scourge of marine fish and shellfish culture (Austin and Austin, 1993) with the severity of the disease increasing with the expansion of mariculture (Smith, 1988). Vibriosis has been problematic wherever finfish culture occurs in the world. Fortunately, diseases caused by *V. anguillarum, V. ordalii* and *V. salomonicida* have been shown to be amenable to control by vaccination (Newman, 1993).

V. harveyi is a common water-borne bacterium known to cause disease in a wide range of marine animals (Zhang and Austin, 2000). The bacterium is a pathogen of penaeid shrimp

(Karunasagar *et al.*, 1994; Alvarez *et al.*, 1998; Robertson *et al.*, 1998), has been implicated in diseases of rock lobster (Diggles *et al.*, 2000) and in a range of fish species including the common snook, *Centropomus undecimalis*, (Kraxberger-Beatty *et al.*, 1990), dentex, *Dentex dentex*, (Company *et al.*, 1999), rainbow trout, *Oncorhynchus mykiss*, and Atlantic salmon, *Salmo salar*, (Zhang and Austin, 2000). The bacterium is possibly implicated in disease in an even wider range of fish species given that another fish pathogen, *Vibrio carchariae*, is now described as a junior synonym of *V. harveyi* (Pederson *et al.*, 1998). The pathogenicity mechanisms of the bacterium have not been fully elucidated, though are thought to involve extracellular products (Austin and Austin, 1993). There is a relationship between siderophore production and virulence in fish (Owens et al., 1996), and Harris and Owens (1999) characterised protein exotoxins from strains pathogenic to penaeid prawns that were lethal to prawns and mice. However, this may be further complicated by the role of bacteriophages in the toxicity of *V. harveyi*. Ruangpan *et al.*, (1999) reported mediation in toxicity of *V. harveyi* to penaeid prawns via transfer of genes controlling toxin production.

As with disease caused by other members of the genus, vibriosis is systemic with fish displaying haemorrhagic lesions, necrosis in the liver (Company *et al.*, 1999) and occasionally exopthalmia and corneal opacity (Kraxberger-Beatty *et al.*, 1990; Company *et al.*, 1999). In Queensland, vibriosis is usually an acute disease affecting fingerling barramundi reared in seacages, with heavy infections resulting in fatalities within 24-48 h (Annette Thomas, pers comm).

1.5 OUTLINE AND AIMS OF THIS THESIS

A starting point in the development of a vaccine against vibriosis caused by *V. harveyi* in barramundi was to examine the immune response of the fish to a bacterin. This was seen as reasonable given the success of inactivated cell cultures or bacterins as vaccines for protection against some forms of vibriosis. An important consideration is the ease and economy of vaccine production and it needs to be established whether a bacterin can be an

effective vaccine. Therefore the overall aim of this study was to examine the humoral immune response of barramundi to a bacterin prepared from *V. harveyi* culture as a potential vaccine candidate. The work carried out for this thesis forms one part of a combined effort within the Cooperative Research Centre for Aquaculture (Aquaculture CRC Ltd., Australia) and field trials evaluating potential vaccines are to be performed by others participants. The findings from this work should form the basis for future work, not only into *in vivo* assessment of prospective vaccines for vibrioisis, but also into more detailed analyses of the barramundi immune system.

Firstly, since no anti- barramundi Ig antisera were commercially available, the investigation required the development of specific reagents capable of measuring the specific humoral or antibody response in barramundi (Chapter 2). Chapter 3 is a general materials and methods section comprising development of appropriate assays to quantify the immune response, preparation techniques of the bacterium as a bacterin and fish husbandry. The first experiment (Chapter 4) attempted to appraise the specific antibody response in the serum and mucus (skin and gut) of barramundi to a bacterin administered with an adjuvant via intraperitoneal (IP) injection or immersion (IMM). As the bacterin had not been administered to barramundi before and there is little or no published information on barramundi humoral responses an adjuvant was used for the injection to be sure of a detectable response. A degree of duplication was unavoidable for the second experiment (Chapter 5) as a different strain of the bacterium had been identified at this time as being more pathogenic and as a candidate for commercial vaccine and was subsequently used in the trial. Having established the ability of the bacterin to elicit a systemic antibody response by IP injection, with adjuvant, and IMM the second experiment attempted to confirm observation of an apparent anamnestic response without adjuvant. This experiment also compared bacterin administration techniques of IP injection, IMM and anal intubation (AI) as simulation of oral antigen delivery to gut-associated lymphoid tissue. With a view to the possibility of developing broadly cross-protective vaccines, the final experiment (Chapter 6) attempted to elucidate the immunogenicity of lipopolysaccharide (LPS) extracted from *V. harveyi*. Lipopolysaccharide has been postulated as the protective antigen in other Vibrio vaccines and may have the potential to be a cross-protective antigen. Additionally, LPS may

be an appropriate candidate for oral administration as the polymer may be resistant to the enzymatic degradation in the gut. The final discussion (Chapter 7) underlines the major findings and points out the applicability of the results to the development of an effective against vibriosis for barramundi.

CHAPTER 2

REAGENT DEVELOPMENT FOR AN ELISA TO DETECT SPECIFIC ANTIBODY IN VACCINATED AND NON-VACCINATED BARRAMUNDI (*Lates calcarifer*)

2.1 INTRODUCTION

Inherent in research efforts in the field of fish vaccinology is the need to evaluate the effectiveness of prospective vaccines in terms of protection and their ability to stimulate specific immune responses (Hastings, 1988). Experimentally this can be done by pathogen challenge models and the monitoring of the adaptive humoral response. The latter is important for a number of reasons. For example: to determine whether there is a correlation between antibody levels and protection, for a more complete understanding of the immune mechanisms enhanced and, to monitor immunogenicity and the protective nature of individual antigens. If there is a positive correlation between protection and antibody and the antibodies have been demonstrated to be protective, then monitoring antibody production will give an indication that the fish have responded to vaccination and are protected against the disease. Measurement of antibodies specific to vaccine antigens can be used as a means of quality control for specific batches of a vaccine. An essential starting point to monitor antibody production is the development of an ELISA to measure antigen-specific antibody activity.

Antisera able to detect barramundi immunoglobulin (Ig) are required to develop this ELISA. Although mAbs are often used as secondary antibodies in such ELISAs and there are now several available that recognise Ig from various teleosts (Scapigliatti, 1996), polyclonal antibodies, if of sufficient specificity, are commonly used. As neither monoclonal nor polyclonal antibodies to barramundi Ig are readily available the first part of this study reports on the isolation of barramundi Ig and the subsequent production of polyclonal

antisera against the Ig. In doing so purification of barramundi Ig by affinity chromatography using Staphylococcal protein A (SpA) or mannan-binding protein (MBP) as capture ligands are compared as are the subsequent antisera that result from the immunisation of rabbits with both whole Ig molecules and heavy (H) chain or light (L) chain polypeptide components.

2.2 MATERIALS AND METHODS

2.2.1 Purification of barramundi immunoglobulin

Barramundi serum was procured from experimental fish held at the Queensland Department of Primary Industry in Townsville and The School of Aquaculture, University of Tasmania, Launceston. Two affinity chromatography methods were used to partially purify barramundi Ig which was then used to produce antibodies in rabbits. Capture ligands used for each method were MBP procured from rabbits and SpA.

MBP-purified Ig was obtained by using a mannan-binding protein affinity chromatography kit (ImmunoPure® IgM Purification Kit, Pierce, IL, USA) with a 5 mL bed volume. Briefly, serum was dialysed against 4 x 1 L changes of sample buffer (10 mM tris, 1.25 M NaCl, 0.02% NaN_3, pH 7.4) at 4°C. After dialysis the serum was diluted 1:1 with a binding buffer (10 mM tris, 1.25 M NaCl, 20 mM $CaCl_2$, 0.02% NaN_3, pH 7.4). Prior to the introduction of 1 mL of the diluted sample, the affinity column was washed at room temperature with 5 mL of elution buffer (10 mM tris, 1.25 M NaCl, 0.02% NaN_3, 2 mM EDTA, pH 7.4), then equilibrated at 4°C with 20 mL of binding buffer. The serum was incubated on the column for 30 min after which free protein was removed via rinsing with 42 mL of binding buffer. Wash fractions (3 mL) were monitored by measuring optical density at 280 nm in a spectrophotometer (UV-1200 Shimadzu). The column was then returned to room temperature and, after addition of 3 mL of elution buffer, was incubated overnight (>16 h). An additional 42 mL of elution buffer was added to the column and 3 mL fractions

collected. Those with OD_{280} >0.015 were pooled and dialysed against 4 x 1 L changes of phosphate buffered saline (PBS; 5 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.2).

SpA-purified Ig was obtained using an Econo-Pac protein kit® (Bio-Rad) with an affinity column, 2 mL bed volume, and buffers supplied or, via a protein A-sepharose matrix, 1 mL bed volume, housed in a column using procedures and buffers described by Bollag *et al.* (1996). In the latter method barramundi serum was diluted 1:1 with starting buffer (100 mM tris-HCl, 100 mM NaCl, pH 7.5) then 0.2 μm filter sterilised. One millilitre of sample was applied to the column, overlaid with 0.4 mL of starting buffer and incubated for 30 min at room temperature. The column was then washed with 15 mL of starting buffer and OD_{280} of wash fractions monitored for contamination before application of 10 mL elution buffer (1 M glycine-HCl, pH 2.5) and incubation for 30 min. Eluent was collected as 1 mL fractions in tubes containing 0.1 mL of neutralising buffer (1 M tris-HCl, pH 8.0). Collected fractions with OD_{280} >0.2 were pooled then either extensively dialysed against PBS or buffer exchanged through a desalting column previously equilibrated with PBS.

Protein content of each Ig preparation was determined with a protein assay kit (Bio-Rad) using bovine gamma globulin (Bio-Rad) as a standard and purity was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Where necessary, Ig in each preparation was concentrated using a centrifugal filter device (Ultrafree-15®, Millipore).

2.2.2 Polyacrylamide gel electrophoresis

Proteins in all Ig preparations were partially characterised by polyacrylamide gel electrophoresis (Laemmli, 1970) under both non-reducing and reducing conditions to determine purity, isomeric state and approximate molecular weights of native and reduced Ig molecules and components. Native preparations were diluted 4:1 in sample buffer (321.5 mM tris, 50% glycerol, 0.05% bromophenol blue) and electrophoresed on 4 or 5% resolving gels with 3% stacking gels. Reduced samples were diluted at the same ratio in reducing sample buffer (60 mM tris, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1%

bromophenol blue), heated to 100°C for 4 min then electrophoresed on 8 or 10% resolving gels with 4% stacking gels both containing SDS. All native samples were electrophoresed in buffer (25 mM tris, 192mM glycine) for at least 1 h with a current limit of 60 mA and at 200 V. Reduced samples were electrophoresed for a similar time using the same buffer but with the addition of 0.1% wv^{-1} SDS. Protein bands on gels were visualised after staining with either Comassie brilliant blue (CBB) (0.5% CBB, 50% methanol and 10% acetic acid) for 1 h, then destained overnight in 10% methanol and 10% glacial acetic acid, or silver nitrate. Silver stain procedures were modified from those of Harlow and Lane (1988). Briefly gels were fixed with 50% ethanol and 10% glacial acetic acid for at least 30 min followed by 15 min in 5% ethanol and 1% glacial acetic acid, rinsed 3 times in distilled water then bathed in 0.2% sodium thiosulphate for 1 min. Gels were exposed to 0.2% silver nitrate with 0.15% formalin solution, rinsed in distilled water then developed in a solution of 6% sodium carbonate, 0.05% formalin and 0.2% sodium thiosulphate. Development was stopped with 1.5% sodium EDTA.

Molecular weights (MW) of heavy and light chain polypeptide components were determined from a plot of the log of molecular weights of known standards against the relative mobilities (Rf) of these standards, where Rf is the distance migrated by the protein divided by the distance migrated by the dye (Hames and Rickwood, 1990). Reference standards were broad range molecular weight markers (Bio-Rad) or multicoloured standards (Novex) and at least 3 gels were used for each determination. For analysis of the whole molecule under native conditions standards were high molecular weight markers (Pharmacia) and human IgM donated from the Immunobiology Unit, University of Technology Sydney (UTS).

2.2.3 Gel filtration

Molecular weight of the whole barramundi Ig molecule was determined by gel filtration chromatography in a Sephacryl high resolution (S–300 gel type; Pharmacia) column with a fractionation range of 10–1500 kDa. Reference standard proteins were bovine serum albumin (66 kDa; Sigma), alcohol dehydrogenase (150 kDa; Sigma), apoferritin (443 kDa;

Sigma), thyroglobulin (669 kDa; Sigma) and human IgM (950 kDa; UTS). The column void volume (Vo) was 92 mL and 2 mL fractions were collected, the elution volume (Ve) was determined from the peak OD at 280 nm. The actual mass of the barramundi Ig molecule was estimated from a standard curve of logarithm of MWs of the standards against their Ve divided by Vo of blue dextran.

2.2.4 Production of rabbit anti-barramundi immunoglobulin serum

Rabbits were housed at the Central Animal House of the University of Tasmania, Sandy Bay Campus, Hobart. Duplicate rabbits were each injected intramuscularly at multiple sites with MBP-purified preparations of barramundi Ig (100 μg protein) diluted in sterile PBS (pH 7.2) with an equal volume of Montanide® ISA-50 adjuvant as a 1.5 mL inoculum. Rabbits were boosted at days 28 and 62 as per initial injection. Blood samples from the lateral ear artery were taken prior to each injection and 30 days after the final booster. Rabbits that had been inoculated with MBP-derived Ig were given additional boosters of H and L chain Ig bands excised from SDS-polyacrylamide gels of MBP-derived product at days 180 and 202 then euthanased and bled at day 216. These additional boosters were prepared by running the MBP-derived product on 10% reducing polyacrylamide gels, excising the bands corresponding to H and L chains, then homogenising with saline and mixing with an equal volume of montanide adjuvant. They were administered as 1.7 mL inocula (approximately 50 μg protein per inoculum) to each rabbit. These boosters were given in an attempt to selectively elicit antibodies directed against barramundi Ig.

SpA-derived Ig (approximate protein concentration of 0.7 mg mL^{-1}) was administered to duplicate rabbits as H or L chain Ig components excised from 10% reducing polyacrylamide gels of the PA product. Excised bands were dialysed against 4 x 1 L changes of PBS over 3 days. Each rabbit was given a 1 mL inoculum containing approximately 112 μg protein (H chain) or 28 μg protein (L chain) with equal volumes of montanide adjuvant. Injections were performed on days 0, 47 and 70. All rabbits were euthanased and bled on day 152. Resultant antisera are referred to as MBP 3 or 4, HC 1 or 2 and LC 1 or 2 with numbers denoting individual rabbits.

2.2.5 Immunoblotting

Western blots were performed to assess specificity for barramundi Ig and cross reactivities with serum proteins from other teleost species. Firstly, either whole barramundi serum or SpA-purified barramundi Ig was diluted in a reducing sample diluent to approximately 100 μg mL^{-1} protein, then proteins separated on SDS-PAGE (10% resolving gel and 4% stacking gel) under reducing conditions. The proteins were electrotransferred onto 0.45 μm nitrocellulose membrane (MFS, USA) in transfer buffer (tris 48 mM, glycine 39 mM, methanol 20% vv^{-1}) using a Semiphor® semi-dry transfer unit with a maximum current setting of 0.8 mA cm^{-2} and a voltage limit of 60 for 1-2 h. Success of protein transfer was confirmed using a temporary total protein stain (0.1% ponceau S wv^{-1} and 5% glacial acetic acid vv^{-1}; Sigma). Various lanes were then cut from membranes as strips to enable individual staining or probing with a range of dilutions of antisera. Temporary stains were removed by rinsing nitrocellulose strips in tris buffered saline (TBS; 20 mM tris, 500 mM NaCl, pH 7.5) for 5 min. Molecular weight standards were permanently visualised after staining with amido black (0.1% amido black 10B, 25% isopropanol, 10% glacial acetic acid) for 1 min then destaining (25% isopropanol, 10% glacial acetic acid) for 30 min (Gershoni and Palade, 1982). Residual binding sites on nitrocellulose strips containing sera or Ig components were firstly blocked with 1% skimmed milk in TBS for 1 h, then washed with TBS, TBS-0.05% tween 20, then TBS again (5 min for each wash). Membranes were immersed in various antisera diluted in blocking buffer (1% skimmed milk in TBS) for 1 h. After repeating the washing step, membranes were immersed in goat anti-rabbit IgG (whole molecule) conjugated to horseradish peroxidase (Sigma) diluted 1:1000 in blocking buffer for 1 h. The washing step was repeated before reactive bands were visualised with 3,3' diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate (Sigma). All steps were carried out at room temperature, and rabbit pre-bleeds in lieu of antisera were used as controls. In an effort to increase its specificity antiserum MBP 3 was applied to the SpA affinity column using the protocol described in section 2.2.1, it was then used to probe barramundi serum and Ig via immunoblots. The same electrophoresis and probing protocols were used to assess cross-reactivites of various antisera with serum constituents of other species including: black bream, *Acanthopagrus butcheri,* southern bluefin tuna, *Thunnus maccoyii*, carp, *Cyprinus*

carpio, rainbow trout, *Oncorhynchus mykiss*, flounder, *Rhombosolea tapirina* , and Atlantic salmon, *Salmo salar*.

2.2.6 Flow Cytometry and immunofluorescence

Flow cytometry was used to assess the ability of various anti-barramundi Ig antisera to recognise lymphocytes with surface Ig (Ig+). Firstly, blood was obtained from a single 4.5 kg adult barramundi held at the School of Aquaculture, University of Tasmania, Launceston via caudal puncture and collected in sodium EDTA tubes. Peripheral blood leucocytes were isolated from whole blood using density gradient centrifugation using a separation medium (Percoll®, Pharmacia) at densities of 1.035 g mL^{-1} layered onto 1.065 g mL^{-1} at 400 x *g* for 30 min at room temperature. Leucocytes were collected at the interface of each density layer then twice washed in cell culture medium (RPMI®, CSL Ltd.) by centrifugation for 10 min at 200 x *g*. Supernatant was then drawn off and cells were resuspended in 1 mL of medium. An aliquot of the suspension, mixed 1:1 with 0.5% solution of trypan blue, was examined microscopically for cell viability and enumeration. Cell densities were adjusted to 4 x 10^8 cells mL^{-1} and the suspension was divided as 0.5 mL aliquots to eppendorf tubes. Tubes were centrifuged for 5 s, supernatants removed and 50 µL of each antisera, diluted to 1:100 in cell culture medium supplemented with 0.1% fetal calf serum (Sigma) and 0.1% NaN_3 (hereafter referred to as staining medium), was added to individual tubes before incubation at 4°C for 30 min. Cells were washed 3 times in staining medium before resuspension in fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Immunotech, France) diluted 1:100 in staining medium and incubated as described before. Washing procedures were repeated 3 times before final resuspension of cells in staining medium containing 1% paraformaldehyde. Controls included the unmanipulated cell suspension, tubes containing a rabbit pre-bleed in lieu of anti-barramundi Ig antiserum and tubes containing all the reagents except the anti-barramundi Ig antiserum or the FITC-conjugated goat anti-rabbit Ig antiserum. Samples were analysed with a flow cytometer (FACScan®, Becton Dickinson) and gated for cells with both low forward scatter (FCS) and side scatter (SSC) characteristics (i.e. lymphoid cells).

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Standard ELISA methods were followed to detect anti-barramundi Ig antibodies in rabbit antisera or in rabbit pre-bleeds. Microtiter plates (Cellstar®, Greiner Labortechnik) were coated in either barramundi serum or the SpA-derived barramundi Ig as antigens, residual binding sites in wells were blocked with 3% sodium casein in PBS solution before being overlaid with rabbit antisera, then finally overlaid with goat anti-rabbit Ig antiserum conjugated to horseradish peroxidase for detection with *o*-phenylenediamine. Initially, to optimise working dilutions, rabbit anti-barramundi Ig sera were titrated against SpA-purified barramundi Ig in typical chessboard titrations (Crowther, 1995). Firstly, antigens in coating buffer (50 mM sodium hydrogen carbonate, pH 9.5) were applied to a 96 well microtiter plate in a 2-fold serial dilution across 12 columns, starting at a protein concentration of approximately 40 μg mL^{-1} for the SpA-derived Ig. After incubation overnight at 4°C excess fluid was removed from wells by flicking and firmly tapping inverted plates on a paper towel and all unbound sites in wells were then blocked with 3% casein in PBS during incubation for 1 h at 37°C. Plates were then washed 4 times with PBS buffer using an plate washer (Immunowash®, Bio-Rad) before the addition of antisera, diluted in blocking buffer (1% casein in PBS), down the rows in a 2-fold serial dilution beginning at 1:50 and incubated for 1 h at 37°C before washing as above. Goat anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate (Sigma) diluted to 1:2000 in blocking buffer was then added to all wells and plates were again incubated for 1 h at 37°C. Following a final wash step, the horseradish peroxidase was activated with 0.05% *o*-phenylenediamine (OPD) free base (Sigma) solution in sodium citrate phosphate buffer (0.1 M citric acid, 0.09 M Na_2HPO_4, pH 5) with 0.012% H_2O_2. The reaction was stopped with 3 M (3 N) HCl after 15 min and optical density (OD) read with a microplate reader (Titertek, MS212) at 492 nm. All reagents were added to wells in volumes of 50 μL. Chequerboard titrations were repeated using SpA Ig starting at 10 μg mL^{-1} protein as coating antigen and antisera at starting dilutions at 1:20. Once the minimum antigen concentration giving a significant OD_{492} (around 1.5-2.0) was determined, all antisera were titrated in duplicate against this antigen concentration using the standard method as described above.

2.3 RESULTS

2.3.1 Purification of barramundi Ig

Yields of barramundi Ig from each isolation method were varied (Table 2.1). The affinity purified products had approximate protein concentrations between 34-58 μg mL^{-1} (MBP-derived product) and 200-700 μg mL^{-1} (SpA-derived product). The large range in protein concentrations of both products was possibly caused by differences in Ig levels of the serum samples used, variation in ambient laboratory conditions and multiple use of the columns. From the elution profiles it can be seen that barramundi Ig was eluted from the MBP column generally in the first 9-12 mL of the elution buffer, whereas the majority of Ig was contained in the first 3 mL of eluent from the SpA column (Figure 2.1).

Table 2.1. Protein concentrations and yields of affinity chromatography purified barramundi Ig using either mannan-binding protein (MBP) or protein A (SpA) as capture ligands

Capture ligand	Pooled eluent protein concentration (μg mL^{-1})
MBP	45
	40
	34
	58
PA	227
	700

2.3.2 Gel electrophoresis and gel filtration

Polyacrylamide gels of reduced Ig products (Figure 2.2A) show that both PA- and MBP-purified Ig contain 3 common bands at approximate MW of 108.4 ± 3.5 kDa, 86.0 ± 5.8 kDa and 24.2 ± 2.0 kDa, n = 3. MBP-purified Ig also contains a fourth band at 55.5 ± 2.7 kDa. PA-purified Ig electrophoresed under non-reducing conditions (Figure 2.2B) showed that the complete Ig molecule forms one protein band suggesting a tetrameric structure in common with other teleost Ig and with a probable with a MW notably higher than 669 kDa. This was confirmed by gel filtration which showed that native Ig molecule had an approximate MW of 929 kDa (Table 2.2).

Table 2.2 Molecular weight (MW) of barramundi Ig estimated from a standard curve of the logarithm of MWs of standard proteins against the elution volume (Ve) divided by the void volume (Vo) of blue dextran after gel filtration. The linear equation for the standard curve was $y = -2.2068x + 5.3187$, $r^2 = 0.9402$ and Vo was 92 mL

Standard	Ve	Ve/Vo	Log MW	MW (kDa)
Bovine serum albumin	142	1.543	1.820	66
Alcohol dehydrogenase	132	1.435	2.176	150
Apoferrtin	116	1.260	2.646	443
Thyroglobulin	108	1.174	2.825	669
Human IgM	92	1.000	2.978	950
Barramundi Ig	98	1.065	2.968	929

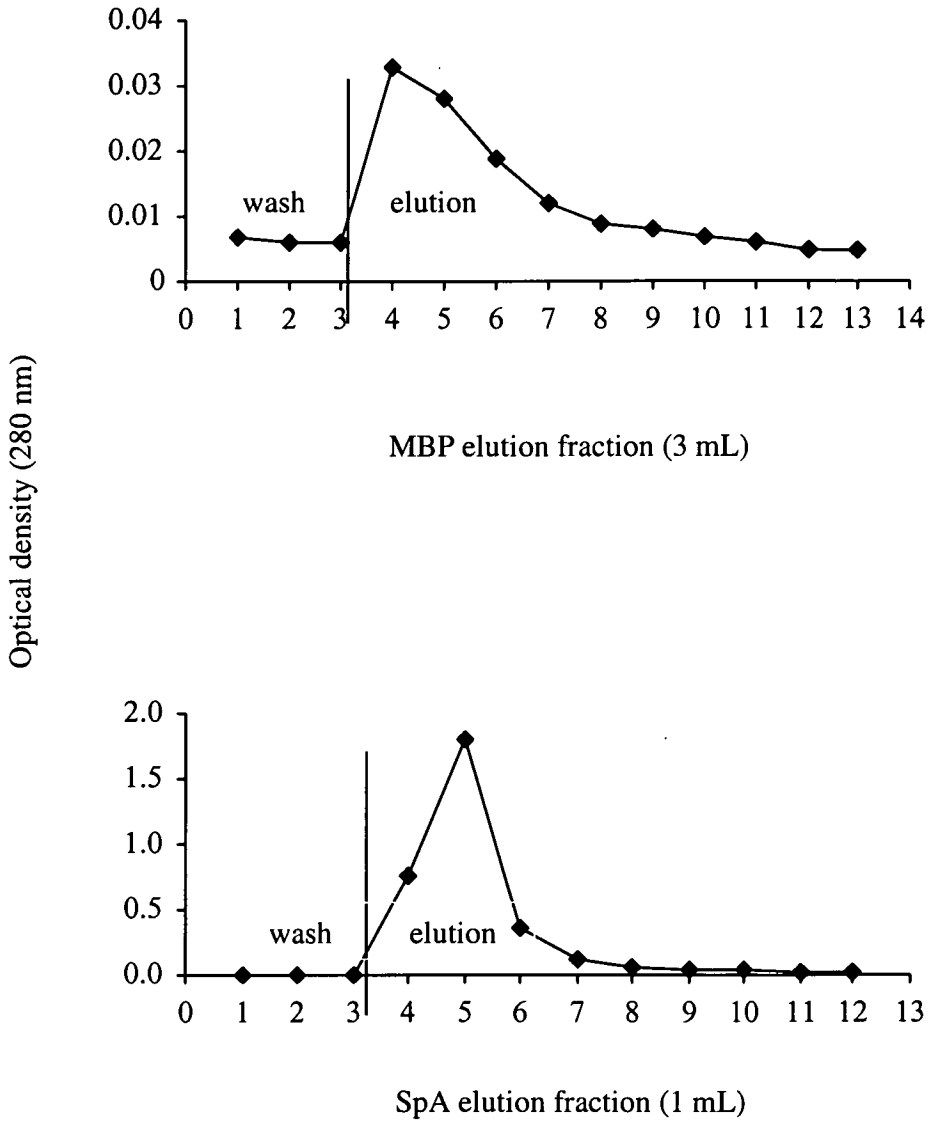

Figure 2.1 Elution profiles of barramundi Ig from both the mannan-binding protein (MBP) and Staphylococcal protein A (SpA) affinity chromatography columns

2.3.3 Immunoblotting

Western blots confirmed the specificity of all antisera, except LC 2, pith reduced barramundi serum components and with reduced SpA-purified barramundi Ig at dilutions up to 1:800 (Figures 2.3-2.5). LC 2 was from a non-responsive animal and this antiserum had no further part in the analyses. When probing reduced SpA purified Ig all antisera were reactive with either or both H and L chain components and two other unidentified bands of approximately 55 and 60 kDa, all rabbit pre-bleeds were also reactive with these bands and, at a dilution of 1:100, MBP 3 was reactive with a protein band which had a molecular weight approximating that of the H chain (Figure 2.6). When probing serum however, rabbit pre-bleeds were negative. Appearance on the blots of a reactive protein bands in the SpA purified Ig and not the barramundi serum suggests that they were contaminants from the SpA column itself, and reactivity with pre-bleeds indicates the possibility of contaminants being SpA. Some leaching of the ligand from the sephadex is not unique (Knicker and Profy, 1991; Godfrey *et al.*, 1992; Steindl *et al.*, 2000) and has been investigated by colleagues in our laboratory (Morrison and Nowak, 2000).

Reactivity with other serum proteins of MBP-derived antisera led to attempts to remove irrelevant antibodies from these samples. This was attempted by adsorption, which tended to remove all antibody activity and, application of MBP 3 through the protein A affinity column. This latter method proved to be relatively effective as subsequent Western blots of the purified antisera (Figure 2.7) show that at a 1:200 dilution reactivity was restricted to the heavy chain component of the barramundi Ig and thus this antisera may be a useful ELISA reagent.

Though background reactivity of HC 1 and 2 and LC 2 antisera was noted with serum proteins other than Ig, this was removed at dilutions higher than 1:400. Therefore, these antisera are potentially useful in immunoassays as markers for barramundi Ig.

Investigations into cross-reactivity of various antisera with serum proteins from other fish species showed consistent activity with southern bluefin tuna (Figures 2.8-2.10). Moieties in

the tuna serum corresponding in molecular weight to H and/or L chain Ig are clearly visible after probing with MBP 3, HC 2 and LC 1. Additionally MBP 3 appears reactive with similar moieties in rainbow trout serum and MBP 4 seems reactive with both the rainbow trout and Atlantic salmon H chain moiety.

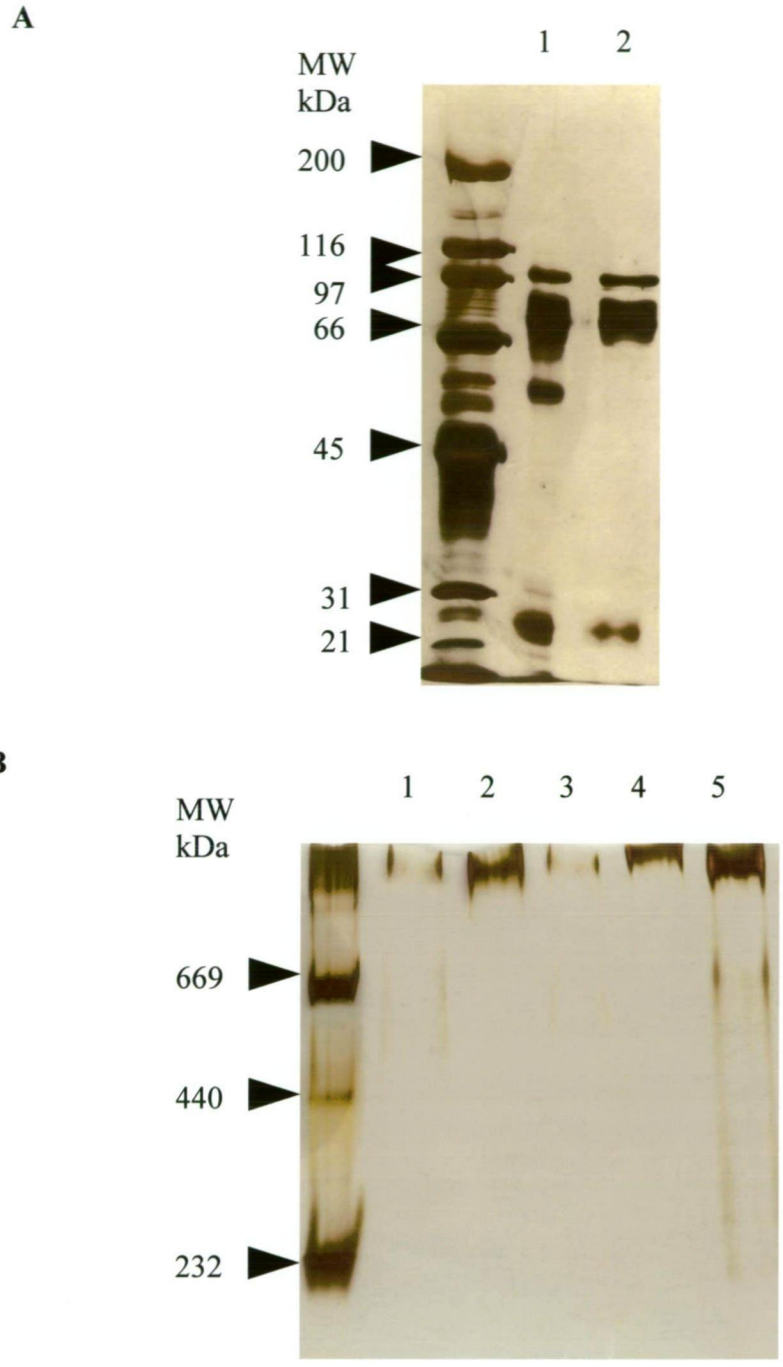

Figure 2.2 Electrophoretic profiles of heterologous Ig. **A.** SDS-PAGE analysis in 10% gel under reducing conditions comparing MBP-purified barramundi Ig (lane 1) with SpA-purified barramundi Ig (lane 2). **B.** PAGE analysis of native Ig molecules on a 5% gel under non-reducing conditions comparing MBP-purified barramundi Ig (lane 1), snapper Ig (lane 2), SpA-purified barramundi Ig (lane 3), human IgM (lane 4) and Atlantic salmon Ig (lane 5). Both gels were silver stained

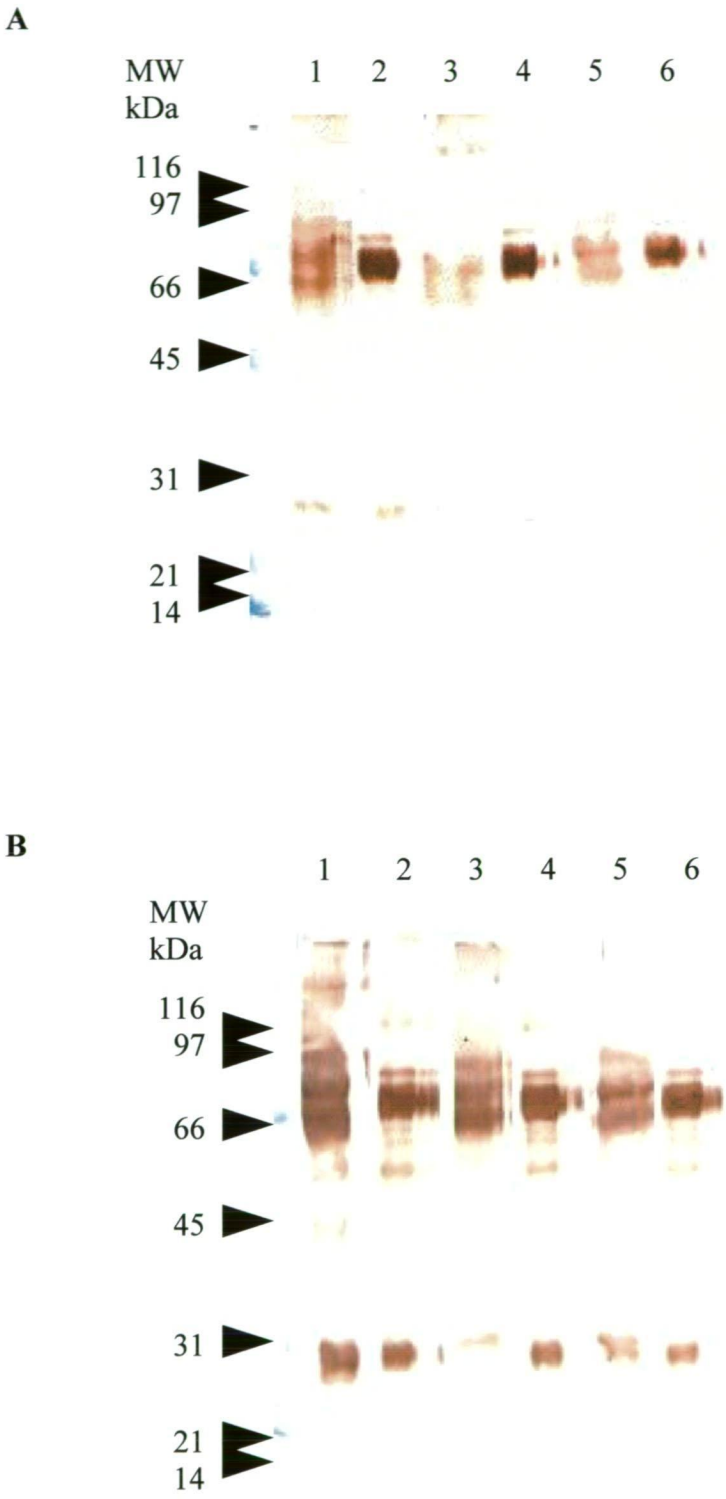

Figure 2.3 Western blot analysis of rabbit anti-barramundi Ig antisera showing reactivity with heavy chain components of reduced SpA-purified barramundi Ig (lanes 2, 4 &6) and barramundi serum (lanes 1, 3 & 5). Probing antisera dilutions were 1:200 (lanes 1 & 2), 1:400 (lanes 3 &4) and 1:800 (lanes 5 & 6). **A.** MBP 3 **B.** MBP 4

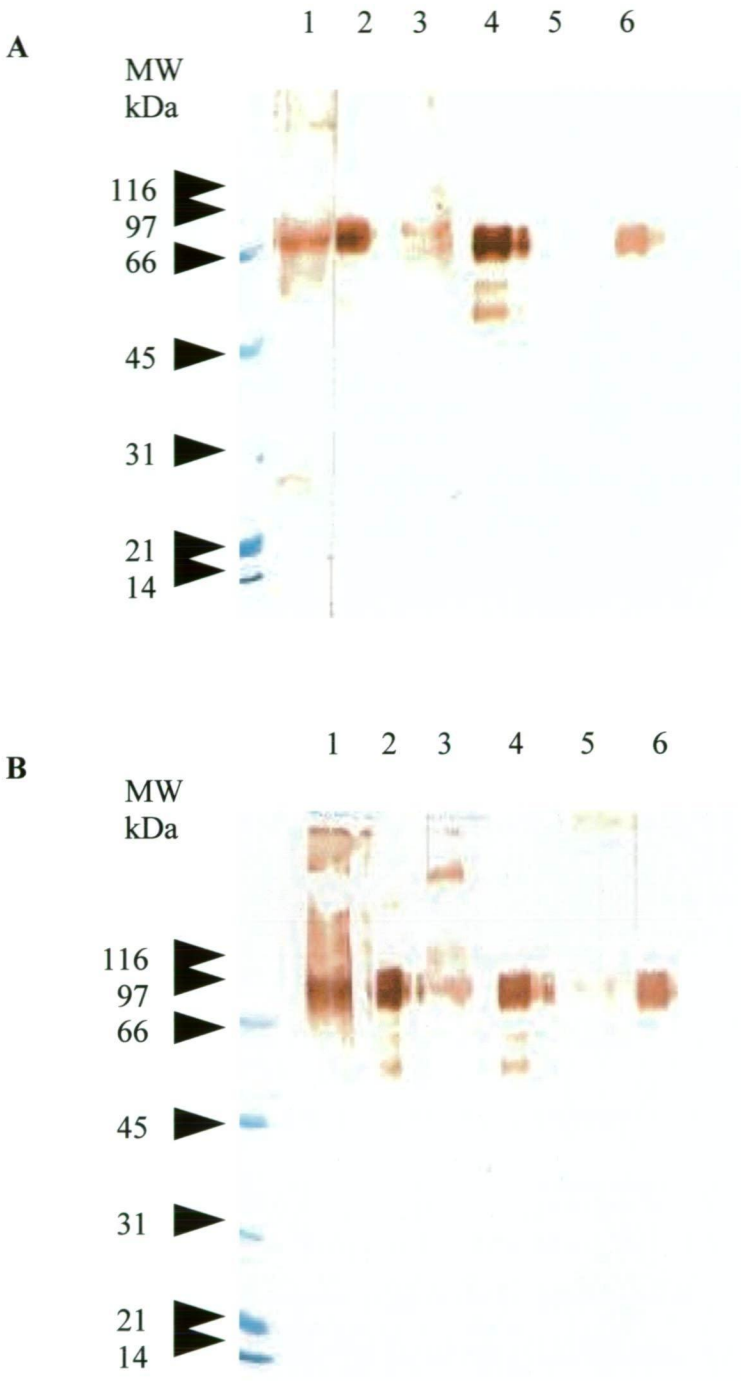

Figure 2.4 Western blot analysis of rabbit anti-barramundi Ig antisera showing reactivity with heavy chain components of reduced SpA-purified barramundi Ig (lanes 2, 4 &6) and barramundi serum (lanes 1, 3 & 5). Probing antisera dilutions were 1:200 (lanes 1 & 2), 1:400 (lanes 3 &4) and 1:800 (lanes 5 & 6). **A.** HC 1 **B.** HC 2

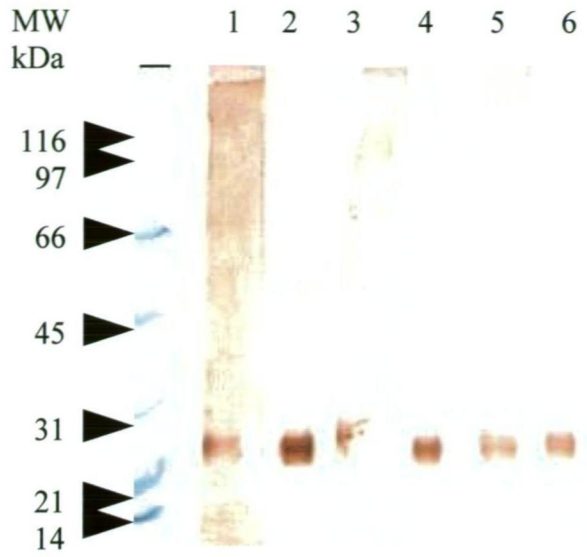

Figure 2.5 Western blot analysis of rabbit anti-barramundi Ig antisera (LC 1) showing reactivity with heavy chain components of reduced SpA-purified barramundi Ig (lanes 2, 4 &6) and barramundi serum (lanes 1, 3 & 5). Probing antisera dilutions were 1:200 (lanes 1 & 2), 1:400 (lanes 3 &4) and 1:800 (lanes 5 & 6).

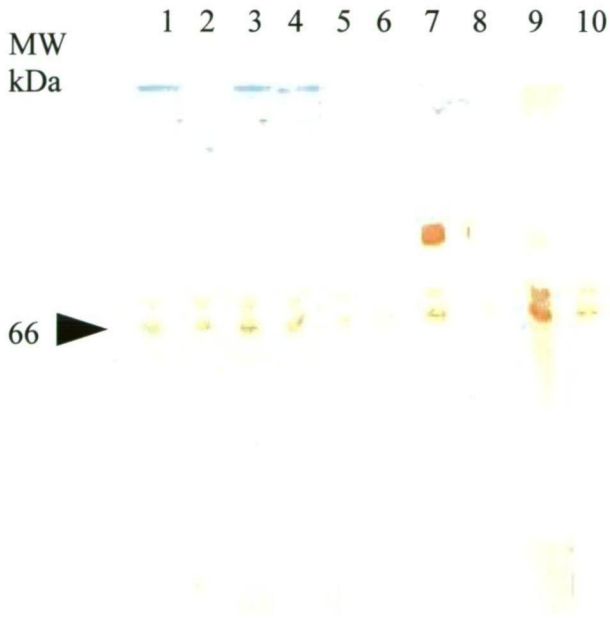

Figure 2.6 Western blot analysis of rabbit pre-bleeds showing reactivity with SpA-purified barramundi Ig. Pre-bleeds were 1:100 and 1:200 in consecutive lanes; HC 1 (lanes 1 & 2), HC 2 (lanes 3 & 4), LC 1 (lanes 5 & 6), MBP 3 (lanes 7 & 8) and MBP 4 (lanes 9 & 10)

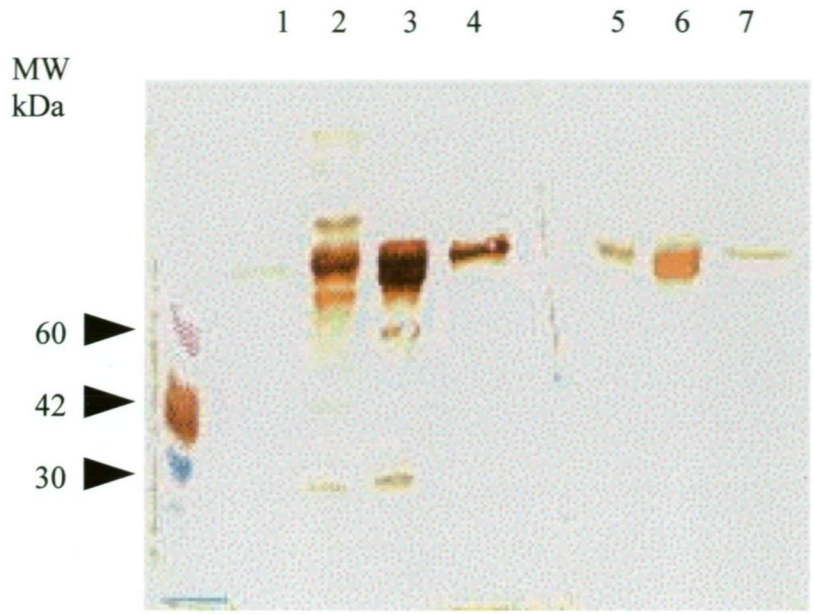

Figure 2.7 Western blot analysis of rabbit anti-barramundi Ig (MBP 3) after application of the antiserum through the SpA affinity chromatography column as an attempt to increase specificity for barramundi Ig. Lanes 2, 3 and 4 contain barramundi serum, SpA-purified barramundi Ig and MBP-purified barramundi Ig respectively and were probed with the antiserum at a dilution of 1:100. Lanes 5, 6 and 7 contain the same serum and purified Ig and were probed with the antiserum at a dilution of 1:200

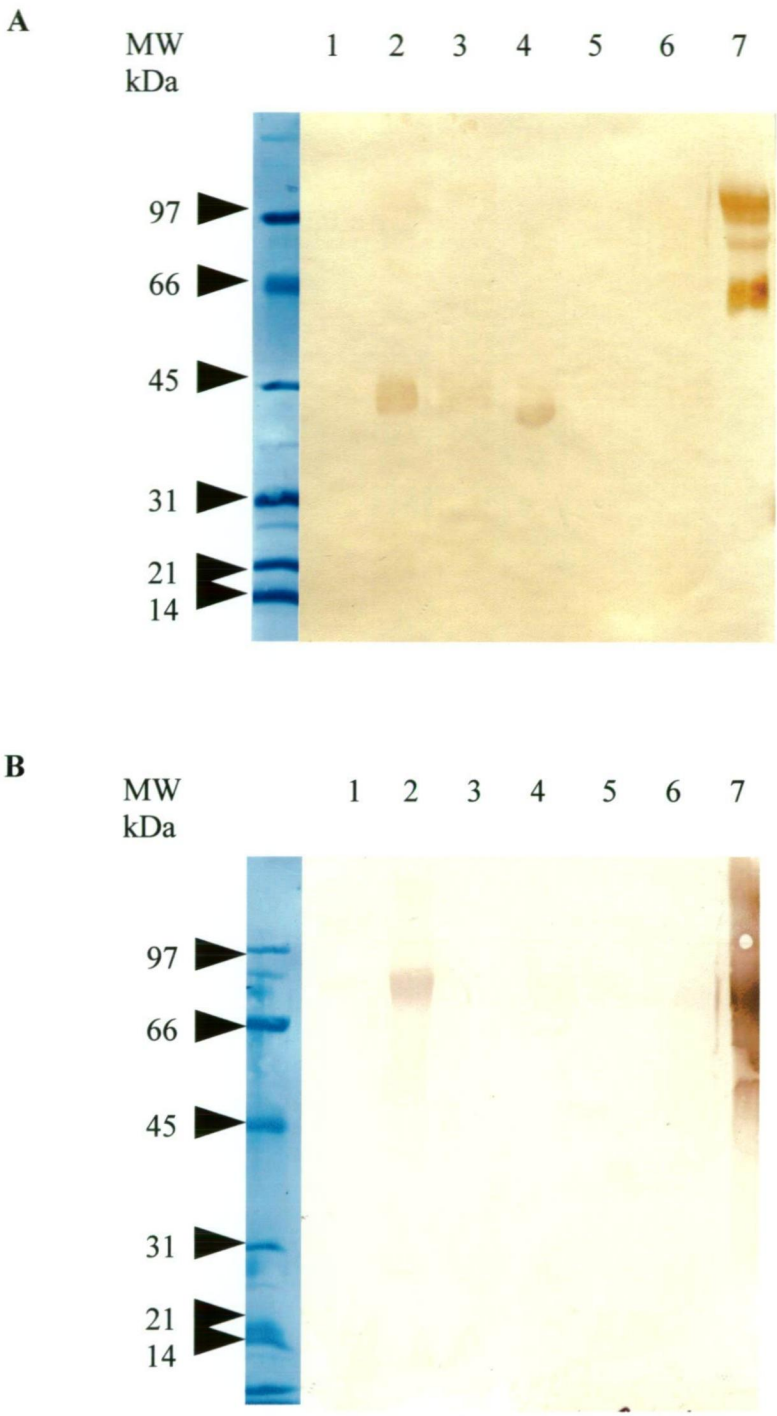

Figure 2.8 Cross-reactivities of rabbit-anti barramundi Ig with heterologous sera, lanes: 1-black bream, 2-southern bluefin tuna, 3-carp, 4-rainbow trout, 5-flounder, 6-Atlantic salmon and 7-barramundi. **A**. Probed with HC 1 at 1:200 **B.** Probed with HC 2 at 1:200

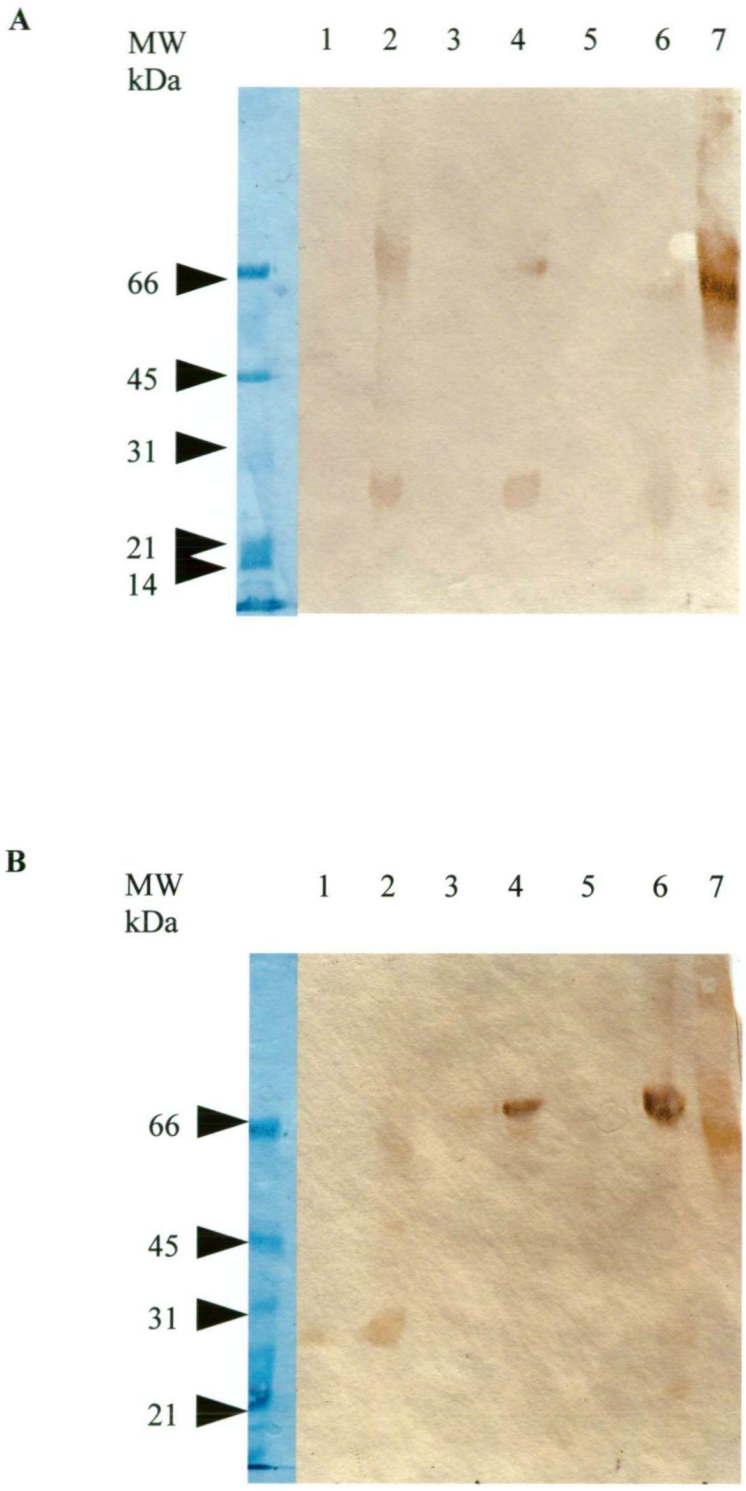

Figure 2.9 Cross-reactivities of rabbit-anti barramundi Ig with heterologous sera, lanes: 1-black bream, 2-southern bluefin tuna, 3-carp, 4-rainbow trout, 5-flounder, 6-Atlantic salmon and 7-barramundi. **A**. Probed with MBP 3 at 1:200 **B.** Probed with MBP 4 at 1:200

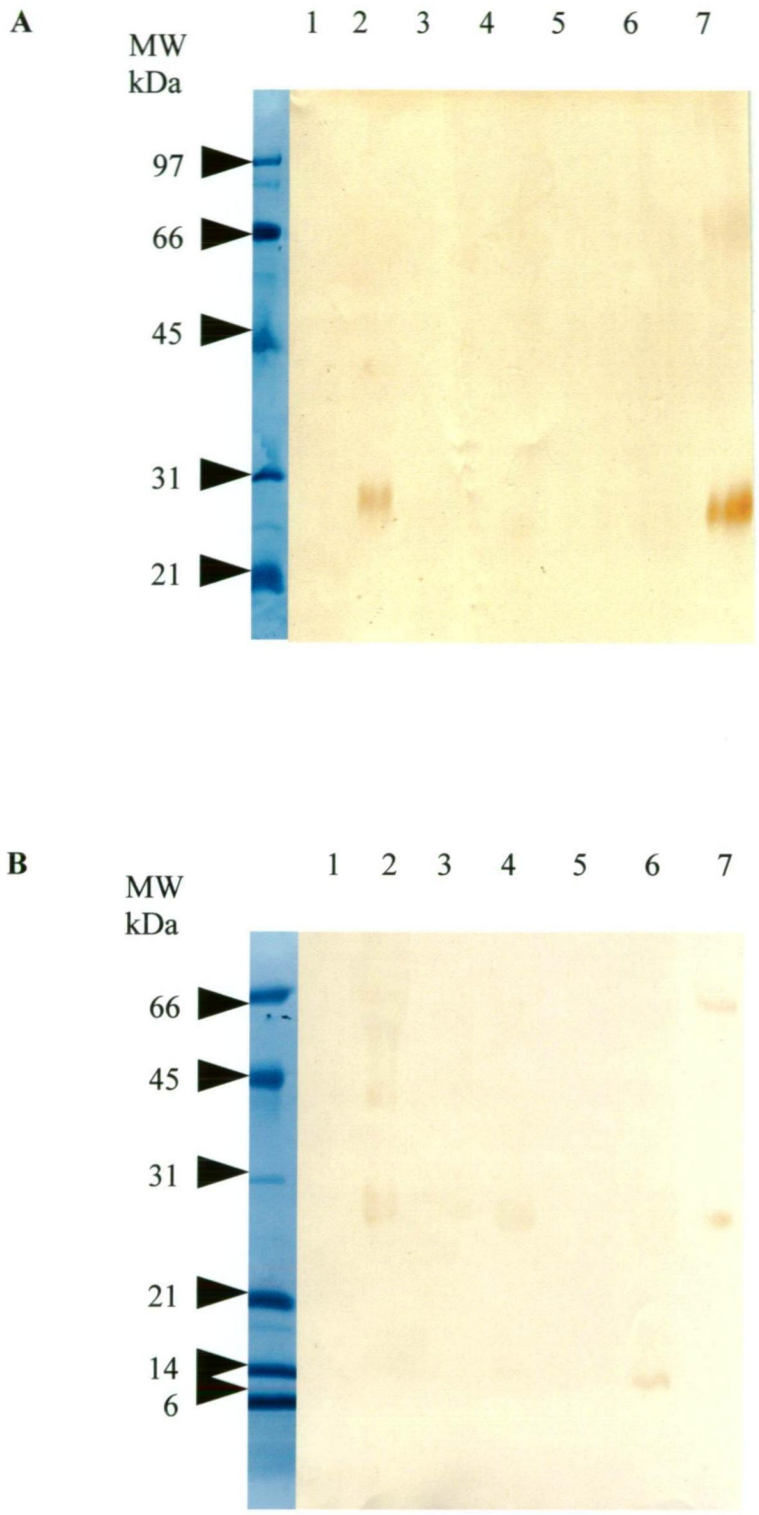

Figure 2.10 Cross-reactivities of rabbit-anti barramundi Ig with heterologous sera, lanes: 1-black bream, 2-southern bluefin tuna, 3-carp, 4-rainbow trout, 5-flounder, 6-Atlantic salmon and 7-barramundi. **A**. Probed with LC 1 at 1:200 **B.** Probed with LC 2 at 1:200

2.3.4 Flow cytometry

Forward scatter versus side scatter profiles and frequency histograms (Figures 2.11 & 2.12) show the gated cell populations analysed and subsequent proportions of fluorescing cells. Much of the apparent debris was unavoidable in this instance due to the time between incubations of cells with antibodies and actual cell counting with the cytometer (approximately 8 h). Fluorescence of proportions of gated cells show that all antisera are capable of binding to lymphocytes, with the percentage of total lymphocytes staining positive varying between each reagent (Table 2.3). All control cells sets were negative. Antisera HC 1 and 2 seem to show higher degrees of specificity relative to MBP antisera which tended to react with higher percentages of lymphocytes.

Table 2.3. Percentage of total barramundi lymphocytes detected by flow cytometry after incubation with various rabbit anti-barramundi Ig antisera

Antisera	Percentage of lymphocytes fluorescing
MBP 4	91.3
MBP 3	73.5
HC 1	61.3
HC 2	52.6
LC 1	69.3

2.3.5 ELISA

The minimum SpA Ig level chosen from the chequerboard titrations for antisera titrations giving OD 492 between 1.5 and 2 was 0.625 μg mL^{-1} (Figure 2.13). Subsequent antisera titrations curves (Figure 2.14) show MBP antisera with substantially higher anti barramundi

Ig activity than either HC and LC antisera. MBP 3 antisera titered out between 40960 and 81920 (reciprocal dilution) whereas HC 1 and LC 1 were between 320 and 640 and HC 2 was between 2560 and 5120. Given the titers and specificities of each antisera SpA-purified MBP 3 and HC 2 were chosen as antisera to use in ELISAs to detect antigen specific barramundi Ig.

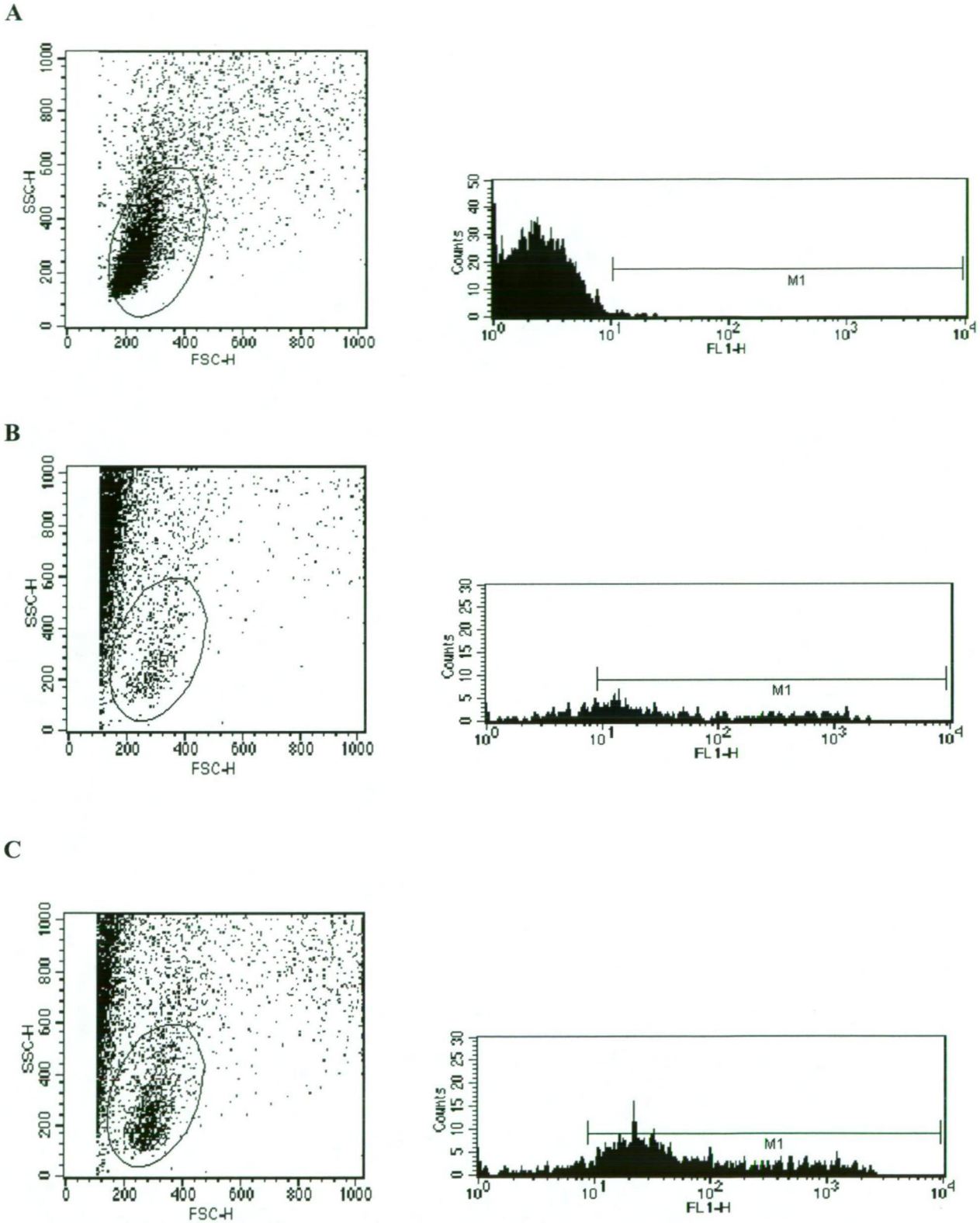

Figure 2.11 Forward scatter/side scatter profiles of peripheral blood leucocytes showing the gated cell population (presumed lymphocytes and frequency histograms indicating FITC+ cells after incubation with various rabbit anti-barramundi Ig antisera and subsequent binding with FITC-conjugated goat anti-rabbit Ig antiserum. **A**. Unmanipulated PBLs **B**. PBLs incubated with MBP 3 **C**. PBLs incubated with MBP 4.

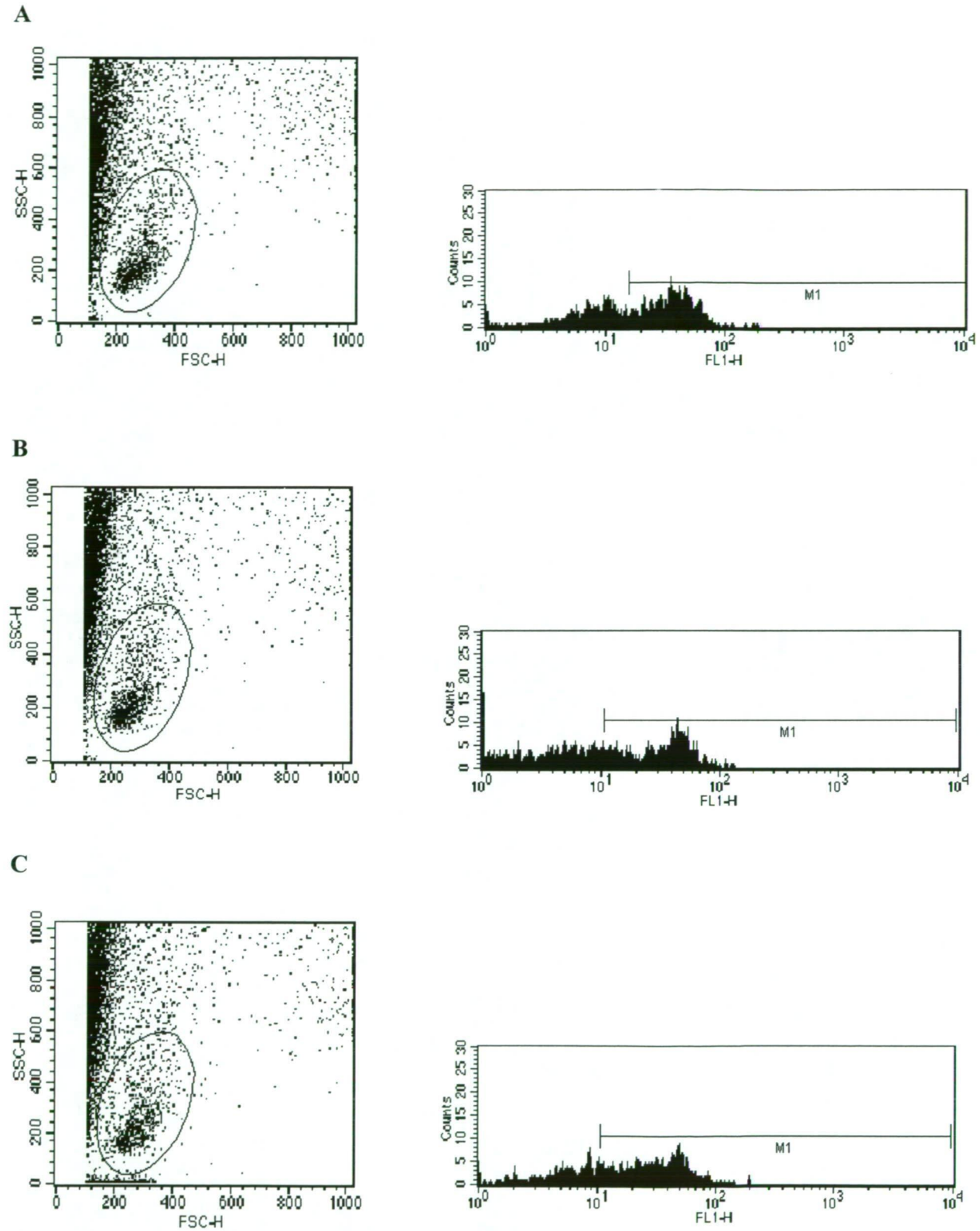

Figure 2.12 Forward scatter/side scatter profiles of peripheral blood leucocytes showing the gated cell population (presumed lymphocytes and frequency histograms indicating FITC+ cells after incubation with various rabbit anti-barramundi Ig antisera and subsequent binding with FITC-conjugated goat anti-rabbit Ig antiserum. **A**. PBLs incubated with HC 1 **B**. PBLs incubated with LC 1 **C**. PBLs incubated with HC 2.

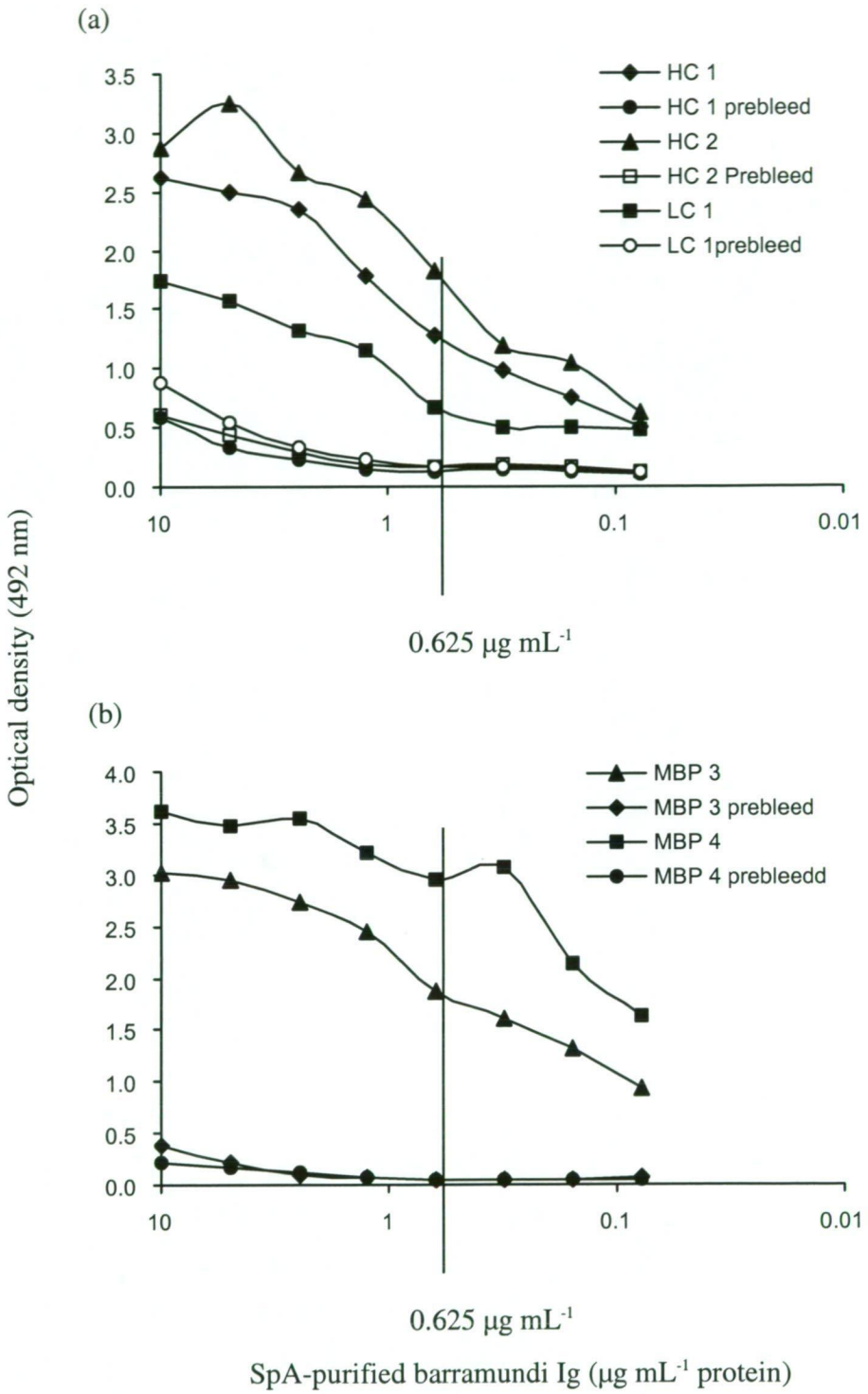

Figure 2.13 Titration curves of SpA-purified barramundi Ig antigen against a single dilution of each rabbit anti-barramundi Ig antisera and pre-bleeds showing 0.625 μg mL^{-1} as the antigen protein concentration chosen to titrate each antiserum. (a) HC 1, HC 2 and LC 1 at 1:20 dilution. (b) MBP 3 and MBP 4 at 1:2560 dilution.

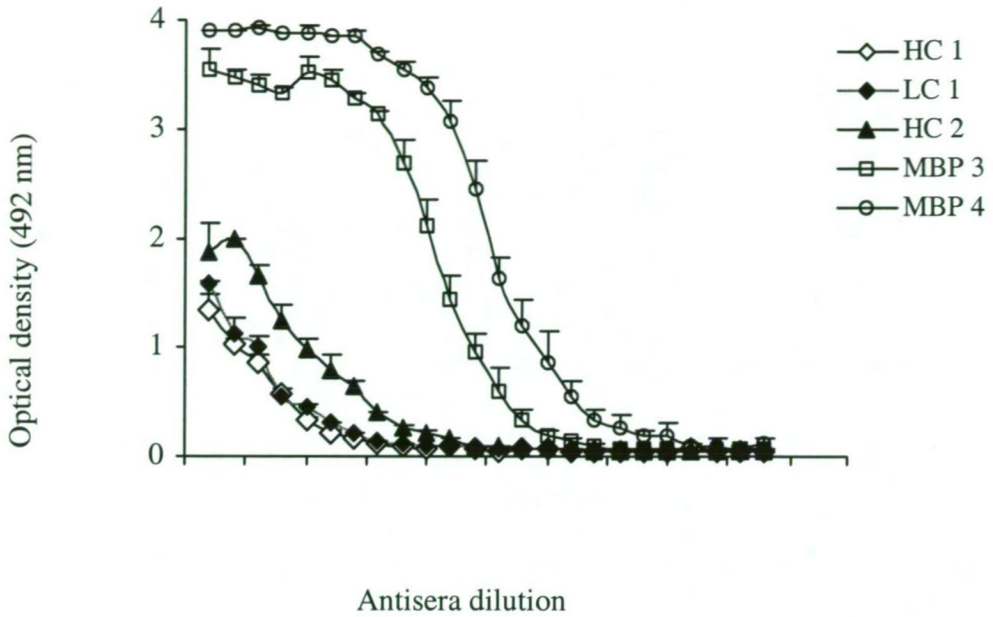

Figure 2.14 Titration curves of various anti-barramundi Ig antisera serially diluted from 1:5 to 1:2.1 x 10^7 against a constant concentration (0.625 μg mL^{-1} protein) of SpA-purified barramundi Ig. Values are means + SE (n = 2).

2.4 DISCUSSION

Purification of the barramundi serum immunoglobulin was most successful in terms of yield and purity using the SpA affinity chromatography column. Affinity chromatography using SpA as the capture ligand has been demonstrated to be effective in isolating IgM from the serum of several fish species including carp, *C. carpio*, (Zikan *et al.*, 1980: Suzuki *et al.*, 1990), goldfish, *Carassius auratus*, yellowtail, *Seriola quinqueradiata*, red sea bream or snapper, *Pagrus auratus*, (Suzuki *et al.*, 1990; Morrison and Nowak, 2000), turbot, *Scophthalmus maximus*, (Estevez *et al.*, 1993; Fournier-Betz, 2000), seabass, *Dicentrarchus labrax*, (Scapigliati *et al.*, 1996), southern bluefin tuna, *T. maccoyii*, (Watts 2000) and several Antarctic fish species (Scapigliati *et al.*, 1997). Conversely, the use of MBP as a capture ligand has had limited use in isolating serum Ig from fish, although it has been used to purify IgM from rainbow trout, *O. mykiss* ,(Kehrer, 1995) and tomato clownfish, *Amphiprion frenatus*, (Cobb *et al.*, 1998). Reasons for the limited use of MBP are unclear but certainly the comparatively low yield using this ligand to purify barramundi serum Ig noted in this study makes it a less attractive option as a capture ligand. In this case the SpA column resulted in a yield which was in the order of 10 times greater than that achieved from the MBP affinity column and seemed to be a more pure product. Yield variation between batches from the same column may probably be attributed to differences in conditions, Ig levels in serum samples or multiple use, but reasons for yield differences between columns are not so clear.

Although it seems that SpA is more efficient in purifying barramundi serum Ig in this study direct comparisons of the IgM binding ability of the two ligands cannot be made mainly because of different column capacities and variation of serum samples. Regardless of the efficiencies of either ligand both have been shown to suitable and relatively simple methods for the isolation of barramundi Ig. It was important to investigate binding ability of these proteins with barramundi Ig as SpA at least has variable capacity to bind with Igs from different fish genera (Estevez *et al.*, 1993).

The possible sample contamination of barramundi Ig with SpA was a concern, particularly for *in vivo* administration given its potential toxicity (Besinger *et al.*, 1984), however, for the purpose of this study the contamination is of lesser importance. To produce anti-barramundi Ig in rabbits only protein bands corresponding in MW to H and L chain components of SpA-derived Ig were excised from polyacrylamide gels were used as inocula. SpA-derived Ig was used for molecular weight estimations of the whole molecule and H and L chain components. What does not concur with the theory of SpA leaching into the samples is the absence of bands on the polyacrylamide gels that correspond in molecular weight to those seen on the blots (approximately 60-66 kDa) and further the molecular weight of SpA is around 42 kDa. Molecular weight discrepancies between SpA and the presumed contaminating SpA seen on blots may be a result of coupling of SpA and Ig residues leading to an apparently higher molecular weight. The strongest evidence that SpA was leached from the column used in this study was Western blot analysis of elution buffer without the addition of serum that showed protein bands reactive with rabbit anti-snapper, *P. auratus*, Ig antiserum, rabbit pre-bleeds and HRP-conjugated goat anti-rabbit Ig antiserum (Morrison and Nowak, 2000). This suggests that contaminating proteins had an affinity for mammalian IgG which is a characteristic of SpA (Kronvall *et al.,* 1970).

Molecular weight analysis by SDS-PAGE under reducing conditions and by PAGE under native conditions showed that as with most teleost Ig studied to date barramundi Ig appears to be made up of HC and LC moieties. Although reported MWs are generally in the region of 70-81 and 23-28 kDa for HC and LC respectively for teleosts, in this study gels indicated MWs of 86 and 24 kDa for HC and LC. Compared to MWs previously reported for barramundi of 70 kDa and 24 kDa (Bryant *et al.*, 1999), there is substantial discrepancy with HC. This may be due to differences between samples, protocols or standard reference markers used, or possibly to anomalous migration of the H chain during SDS-PAGE due to high glycosylation as reported by Wilson *et al.* (1985) in goldfish, *C. auratus*. Molecular weight of native barramundi Ig can be confirmed by gel filtration. However, given the approximate values of H and L chains the MW of the native molecule would be approximately 880 kDa. This assumes a tetrameric structure for barramundi Ig which is

characteristic of many teleost Igs (Acton *et al*, 1971; Lobb and Clem, 1981), and was implied by electrophoresis under non-denaturing conditions (Figure 2.2B).

Specificities of the various rabbit anti-barramundi Ig antisera, investigated by Western blots revealed that MBP 3 and 4 were reactive with a number of serum proteins including both those presumed to be the H and L chain components. However, HC 1 and 2 and LC 1 were more specific for barramundi Ig to the point where HC 1 and 2 did not react with the L chain and LC 1 did not react with the H chain suggesting a lack of antigenic similarity between both Ig components. Application of MBP 3 antiserum to a SpA affinity chromatography column improved its specificity and at dilutions of 1:200 was only reactive with the H chain Ig moiety. This process effectively isolated the IgG component of the antiserum and removed any extraneous proteins which may have interfered with immunoassays.

Assessment of cross-reactivity with serum components from other species revealed that HC 2, LC 1 and MBP 3 reacted with components from tuna Ig of the same MW as barramundi HC and LC, i.e. approximately 86 and 24 kDa respectively. Heavy and light chain moieties in purified tuna Ig appear as doublets on polyacrylamide gels with approximate MWs of 71.2 and 74.6 kDa for the H chain and 28 and 29 kDa for L chain respectively (Watts, 2000). This discrepancy in MWs may indicate either that bands visible in the tuna serum are not Ig or, may be due to differences in experimental technique. For example, in this study MWs are inferred from markers transferred to nitrocellulose from one gel only and not replicated gels as reported by Watts (2000). Also, migration patterns of purified Ig may vary from those of Ig in a whole serum environment. In any case, further characterisation of cross-reactivities is required to elucidate the potential value of these antisera in work on other species.

Immunofluorescence measured by flow cytometry demonstrated reactivity of all antisera with sub-populations of gated peripheral blood lymphocytes (PBLs) although percentages of fluorescing cells vary between each. MBP-derived antisera appears to be cross-reactive with T cells with percentages of reactive cells within PBLs (73.5 and 91.3%) higher than for SpA-derived antisera (52.6-69.3%) which is indicative of the higher specificity for Ig of HC

1 and 2 and LC 1. However, common observations made are that mammalian polyclonal antibodies to purified Ig from various fish species are reactive with all fish lymphocytes (Miller *et al.*, 1998). This cross-reactivity has been attributed to the existence of anticarbohydrate antibodies in the antiserum and their reaction with surface glycoproteins and glycolipids on both B and T cells (Yamaga *et al.*, 1978). Whether or not the reactive cells in this study were solely Ig^+ is not certain though percentages of reactive cells in PBL of barramundi elucidated by SpA-derived antisera are, in some cases, only slightly higher than reported for other teleost species when stained with various mAbs such as 46% in rainbow trout, *O. mykiss*, (DeLuca *et al.*, 1983; Sanchez *et al.*, 1993), 35-51% also in rainbow trout (Thuvander *et al.*, 1990) and 21 - 31% in sea bass *D. labrax* (dos Santos *et al.*, 1997). Polyclonal antibodies by definition are not monospecific and therefore cross-reactivity is expected, however, mAbs used in the studies cited above are specific for an individual epitope on the H chain. Given the isotypic variation within both H and L chain Ig constituents in some teleosts (Lobb *et al.*, 1984; Lobb and Olson, 1988) mAbs may not be reactive with all Ig^+ cells thus percentages quoted possibly underestimate proportion of Ig^+ cells in PBLs. In this study flow cytometry was used to assess the ability of anti-barramundi Ig antisera to react with Ig+ cells and thus provide additional evidence as to the specificities of the antisera. Results indicate this to be the case for the SpA-derived antisera. Although some caution in interpretation is required as some Ig^+ cells may be monocytes with serum Ig bound via the Fc receptor (Morrison *et al.* submitted).

ELISA results of the characterisation of various antisera indicate that MBP-derived Ig elicited far greater responses in rabbits than SpA-derived Ig. This may be due to the kinetics of the rabbits' response, individual variation and the slightly variable protein levels in each inoculum, MBP Ig immunised rabbits had a longer time to respond as they were terminally bled at day 216 after initial immunisation compared to day 152 for SpA Ig immunised rabbits. The MBP Ig was also injected into rabbits as the whole product subsequently containing all the impurities, whereas SpA Ig was injected in reduced form as either H or L chain which may explain the better specificity of the SpA Ig-derived antisera.

Given the sensitivities and specificities of all antisera characterised, MBP 3 and HC 2 were chosen for use in immunoassays to detect antigen-specific antibody in barramundi after immunisation.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter details the materials and methods that recur throughout reports of each experiment. Two experiments were carried out where barramundi were immunised with a formalin-killed *Vibrio harveyi* cell suspension (bacterin) via: 1. intraperitoneal (IP) injection or immersion (IMM) and, 2. IP injection, anal intubation (AI) or IMM. In a third experiment barramundi were immunised by IP injection with either bacterin or lipopolysaccharide extracted from *V. harveyi.* Materials and procedures that are unique to a particular chapter or deviate from those described below appear in the relevant sections.

3.2 FISH HUSBANDRY

Barramundi (*Lates calcarifer*) were purchased from West Beach Aquaculture, Wingfield, South Australia and held in a static systems consisting of 140 L glass aquaria containing two airlift pumps and bottom gravel biofilters. Fish were held in seawater (35 ppt) at 25.7 ± 0.5° C (10-12 fish per tank), fed a commercial feed (Gibsons) at 2% body weight per day and acclimated over 4-6 weeks. Temperature and salinity were measured daily and water quality including total ammonia, nitrite, pH and dissolved oxygen monitored every 3-4 days. Tanks were cleaned as required with 50% water exchanges occurring at least every week. Total ammonia levels never exceeded 0.25 mg L^{-1} and if nitrite levels exceeded 2.0 mg L^{-1} water exchanges were carried out, both pH and dissolved oxygen remained relatively constant at approximately 7.3 ± 0.2 and 6.4 ± 1.4 mg L^{-1} (i.e. approximately 100% saturation)

respectively. Although it would be ideal to minimise any possible confounding factors, such as exposure to environmental bacteria of the genus *Vibrio*, by holding the barramundi in freshwater they were held in seawater in accordance with current and future production conditions in Australia where the majority barramundi are reared in seawater (C. Foster pers comm).

Before immunisations, tanks were randomly assigned a treatment via a lottery method and fish were similarly randomly allocated to tanks and weighed at the beginning of each experiment. For any treatment requiring individual handling of fish, ethyl-*p*-aminobenzoate (benzocaine) at 70 mg L^{-1} was used as an anaesthetic.

3.3 BACTERIN PREPARATION AND ADMINISTRATION

Two strains of *V. harveyi* (designated 9056681 and 9050405) were isolated and characterised by Dr Lachlan Harris of James Cook University, Townsville, Queensland and were made available by Dr Jeremy Carson from the Department of Primary Industries, Water and Environment, Mount Pleasant Laboratories, Launceston, Tasmania. Bacteria were grown on Oxoid nutrient broth no.2 (NB2) supplemented with NaCl to 1.5% as 2 L volumes in 3 L flasks on a shaking water bath at 22°C for 72 h or 30°C for 24 h to densities of approximately 10^8 cells mL^{-1} as determined by hemocytometer counts. Cultures were then inactivated with formalin to a final concentration of 0.5%, returned to the water bath overnight (approximately 15 h) and finally stored in sealed sterile bottles at 4°C until required. Immediately prior to inactivation with formalin cultures were plated to horse blood agar to ensure uniform colonial morphology and culture purity. After inactivation sterility was confirmed by incubating 1-2 mL cell suspension aliquots in 15 mL of fresh salt supplemented NB2 medium at 22°C for 4 days then attempting to subculture onto horse blood agar plates. Additional checks were made using plates of the same medium inoculated after cells had been rinsed 3 times in saline. Cell densities of subsequent batches of bacterin were estimated from a standard curve (Figure 3.1) of cell numbers, determined by duplicate

hemocytometer cell counts, versus optical density at 550 nm measured on a spectrophotometer (UV 1200, Shimadzu).

To prepare inocula for IP injection and AI cultures were centrifuged at 1000 x *g* for 15 min and cells harvested then washed twice in sterile saline (0.15 M NaCl) and recollected after centrifugation as above. Harvested wet weight was defined as the wet weight of pellet with supernatant removed. Cells were then suspended in sterile saline to a wet weight concentration enabling administration to fish at a dose approximating 0.08-0.1 mg of cells per g of fish. In the experiments barramundi were administered bacterin or sterile saline placebo via IP injection in volumes ranging between 0.1 and 0.4 mL depending on fish size which ranged from 52-95 g. Anally intubated fish (mean weight 93.6 g ± 14.4 g) were starved for 48 h prior to immunisaton and received bacterin or a placebo as 0.3 mL volumes via a flexible silicon tube attached to an 18 gauge needle and inserted approximately 5 cm along the intestine. The rationale was that bacterin would move further along the intestine on expulsion from the syringe but may also flow back toward the anus on removal of the silicon tube. Some back flow of bacterin from the anus was observed after removal of the tube. Prior to immunisation a single fish was sacrificed and dissected to ascertain length of intestine which was 7.8 cm. For IMM immunisation, inactivated cultures were diluted 1:5 with seawater (35 ppt at 26°C) with no rinsing or harvesting of cells. Pairs of fish in dipnets were immersed for 60 s in 6 L of dilute vaccine (approximately 2 x 10^7 cells mL^{-1}) in a 10 L container with aeration then transferred to a container to rinse off excess bacterin before being returned to holding tanks. To facilitate easy removal of fish from holding tanks, water levels were lowered and 50% water changes carried out. Control fish were handled identically and dipped in 6 L of seawater and then moved to another container as above.

To acquire standard barramundi anti-*V. harveyi* antisera to both strains of the bacterium as positive controls and provide reference specific antibody activity, barramundi were immunised with either strain. For strain no. 9056681, a 4 kg barramundi was IP injected with a 140 mg dose of bacterin in an equal volume of Montanide® ISA-50 adjuvant, then re-immunised 11 weeks later and bled after a further 2 weeks. For strain no. 9050405, six fish (95 ± 12 g) were each IP injected with 8 mg of bacterin in an equal volume of Montanide®

ISA-50 adjuvant, re-immunised after 4 weeks, then serum was harvested after a further 2 weeks.

3.4 SAMPLING PROCEDURES

Prior to sampling fish had been starved for at least 48 h. Fish were lethally anaesthetised with benzocaine at 200 mg L^{-1}, and samples taken as follows. Skin mucus was collected by gently scraping the skin from the operculum to the caudal peduncle with either the blunt edge of a scalpel blade or a cane skewer and immediately placed in 1.5 mL tubes containing 0.5 mL of borate buffer (0.2 M boric acid, 0.05 M sodium tetraborate, pH 7.8) with protease inhibitors [1 mM phenylmethyl-sulphonylfluoride (PMSF), 5 mM iodoacetamide and 0.02% NaN_3], (Ainsworth *et al.*, 1995), then placed on ice. Blood was collected in syringes via caudal puncture using 27 gauge needles or, in blood tubes after severing the tail then allowed to clot at 4°C overnight. The following day serum was drawn off and stored at -20°C until assayed. Due to the occasional difficulty in obtaining blood samples by caudal puncture tail severing was the only means to access blood. Gut mucus was collected after a 5-6 cm section of the intestine, cut approximately 0.5-1 cm anterior to the anus and 1-1.5 cm posterior to stomach, was removed and mesentery tissue gently scraped from the organ. One millilitre of borate antiprotease buffer was then cycled through the section of intestine at least 12 times, collected in 5 mL tubes, then immediately placed on ice. All mucus samples were frozen at -20°C within 8 h of sampling. Dissected kidneys were kept in fish physiological saline (FPS: 0.11 M NaCl, 0.15 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.05 mM KH_2PO_4, 0.12 mM $NaHCO_3$) in ice until macrophage collection.

Some aspects of the sampling procedures require comment. Blood sampling via caudal puncture was, at times, very difficult and often delivered very small volumes which then limited the number of assays which could be performed using serum. This was the case in the experiment described in Chapter 4 and in the circumstances severing the tail was the only means to get blood. This is not ideal practice as water, mucus and urine from the fish

may contaminate the sample and have a diluting effect. Care was taken to prevent contamination by wiping water and mucus from the fish and positioning the blood tube under the severed caudal vein so as to preclude entry of any fluids seeping from the vent. In subsequent experiments caudal puncture via the lateral or ventral surface was a far more consistent means of extracting blood.

Skin and gut mucus samples were collected using simple methods. Although other techniques for collecting cutaneous mucus using filter paper (Lobb and Clem, 1981; Lobb, 1987; Zilberg and Klesius, 1997), cotton wool buds (Jenkins *et al.*, 1994) and, allowing fish to shed mucus after being placed into small plastic bags with water (Wong *et al.*, 1992) have been described, careful scrapping of the skin was a simple and efficient way to collect cutaneous mucus. Previously reported methods for collecting gut mucus include collecting faeces from the intestine (Rombout *et al.*, 1986; Ainsworth *et al.*, 1995), anal intubation (Al-Harbi and Austin, 1992b), gut washings (Ainsworth *et al.*, 1995) and stroking the exposed lumen of the gut wall with cotton buds (Lavelle *et al.*, 1997). In the experiments described here gut mucus was collected by repeatingly flushing a section of intestine with a small volume of buffer, this effectively collected the gut contents, although faeces were absent due to the 48 h fasting period. As fish lack IgA which is resistant to proteolytic degradation after secretion into the gut lumen (Ellis, 1995) both skin and gut mucus samples were stored in buffer containing anti-proteases to prevent any Ig degradation due to proteases in the mucus secretions.

3.5 IMMUNE ASSAYS

3.5.1 Macrophage isolation

Leucocytes were harvested from excised anterior kidneys by firstly pushing kidney material with Leibovitch medium (L-15; Sigma), supplemented with 1 mL L^{-1} barramundi serum, through a fine stainless steel mesh with a syringe plunger then homogenising in 7 mL glass

tissue grinder (Wheaton). Leucocytes were isolated from homogenised material via density gradient centrifugation using a separation medium (Percoll®, Pharmacia) at densities of 1.030 g mL^{-1} layered onto 1.065 g m L^{-1} at 400 x *g* for 30 min at room temperature. Cells were collected at the interface of each layer, washed twice in 3-4 mL of L-15 before final resuspension in FPS. Cell counts and viability were determined using a 0.5% trypan blue solution mixed 1:1 with an aliquot of the cell suspension and a hemocytometer, then cell densities were adjusted to 4 x 10^6 viable cells mL^{-1}.To determine percentages of macrophages cell suspensions were smeared onto glass slides, air dried and stained (Diff Quick®, Pathtech), then differential cell counts performed.

3.5.2 Phagocytic activity

Phagocytic activity of the macrophage suspension was determined using a technique based on that of Seeley *et al.*(1990). Fifty microlitres of the macrophage suspension was incubated with 100 µL of congo red-stained yeast cells on a glass slide. Yeast cells were prepared by hydrating 0.22 g dry yeast with 24 mL phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 3 mM KH_2PO_4, 0.12 M NaCl, pH 7.2) staining with 1 mL congo red, incubating for 5 min then autoclaved for inactivation. Cells were rinsed three times in PBS then the concentration adjusted to 10^8 cell mL^{-1}. After incubation at 22°C for 2 h, the suspension was mixed on the slide and cells were counted at 1000 x magnification. Results are expressed as the phagocytic index (i.e. % of cells containing 1 or more yeast cells) with a minimum of 100 cells counted.

3.5.3 Lysozyme assay

Lysozyme levels in sera samples were measured by a turbidometric assay (Thompson *et al.* 1994) where the activity of lysozyme is determined from a standard curve indicating the degree of lysis of *Micrococcus lysodeikticus* (Sigma) by known concentrations of hen egg white lysozyme (Sigma). One hundred and seventy five microlitres of a *M. lysodeikticus* suspension (75 mg in 100 mL of 0.1 M phosphate/citrate buffer with 0.09% NaCl, pH 6.0) was added to quadruplicate wells of a microtiter plate containing 25 µL of either sera samples or hen egg white lysozyme standards (0-20 µg mL^{-1}). After immediate plate shaking

optical densities at 450 nm were recorded at 5 min intervals in a multiscan plate reader (MCC/340 Titertek Multiskan®) to measure the rate of cell lysis relative to wells containing suspensions of *M. lysodeikticus* only. Optimal pH of 6.0 for the phosphate/citrate buffer was determined after measuring lysozyme in standard barramundi sera and the reference standard (hen egg white lysozyme) over a pH range of 5 to 8 with 0.5 increments (Figure 3.2).

3.5.4 Enzyme-linked immunosorbent assay (ELISA)

The standard indirect ELISA protocol using buffers and equipment described in section 2.2.6 was followed to detect specific antibody in all barramundi serum samples. In this project, two different strains of *V. harveyi* were used for immunisations in different experiments which necessitated ELISA protocols using either strain as appropriate for coating antigens. Similarly, two different rabbit anti-barramundi Ig antisera were used for detection of specific barramundi antibody (MBP 3 and HC 2). *V. harveyi* strain no. 9050405 was used as it superceded strain no. 9056681 in terms of virulence, and a second antiserum was used as it was more specific. Consequently, concentrations of both sets of reagents required optimising before use. The details of these optimisations are described in the Materials and Methods section of the relevant Chapters.

Samples from each experiment were titrated in duplicate between dilutions of 1:50–1:1600 and 1:100-1:3200 and all ELISA plates included both immune and non-immune standards. Anti-*V. harveyi* antibody activities were quantified using the method described by Arkoosh and Kaattari (1992), where the activity of a sample is calculated relative to the activity of the immune standard and is expressed as units of antibody activity per volume of serum. To ensure that the OD was proportional to the reaction rate within each well a kinetic analysis was performed where absorption was measured after the addition of OPD and subsequent development at 450 nm every minute for 15 min prior to stopping the reaction with addition of HCl (Figure 3.3). Thus the development incubation period of 10 min was set.

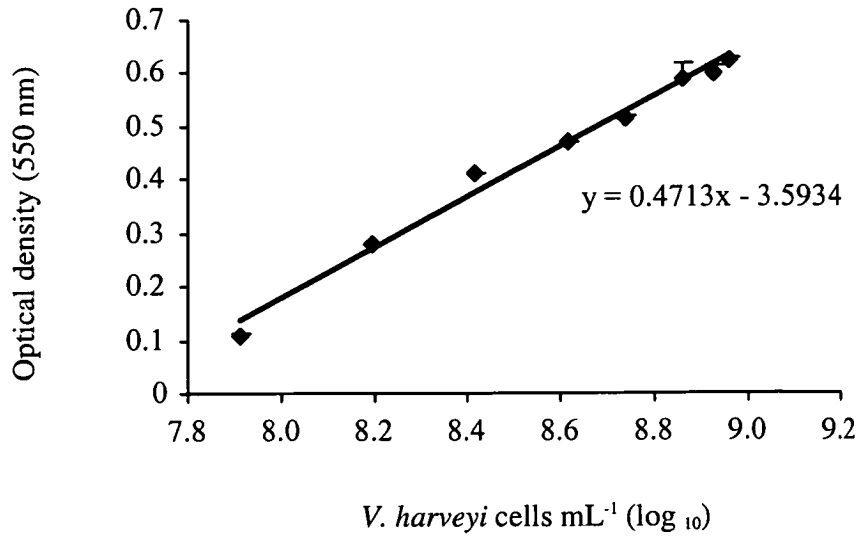

Figure 3.1 Linear regression of absorbance at 550 nm versus *Vibrio harveyi* (strain no. 9050405) cells mL^{-1} ($\log_{10}$). Values are means + SE, n = 2, $r^2 = 0.998$.

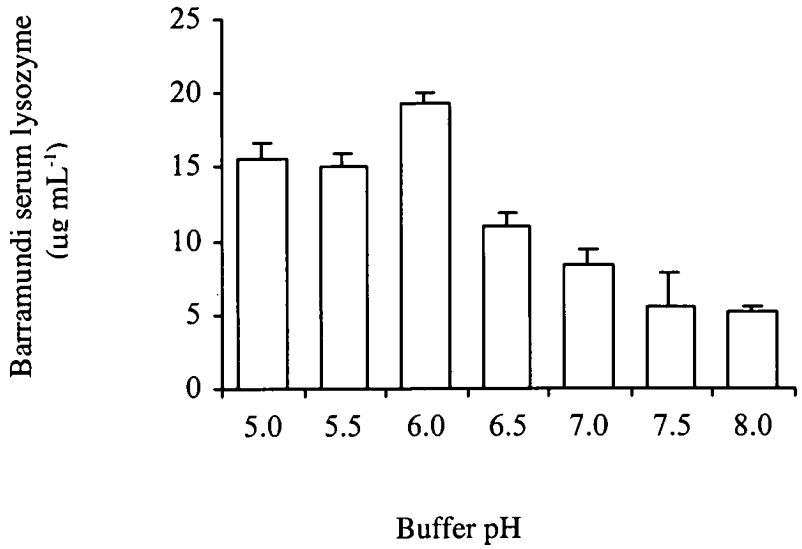

Figure 3.2 Lysozyme activity of barramundi serum measured at a range of pH against hen egg white standard showing maximum at pH 6.0. Barramundi serum was from non-treated fish and was assayed in quadruplicate. Values are means + SE.

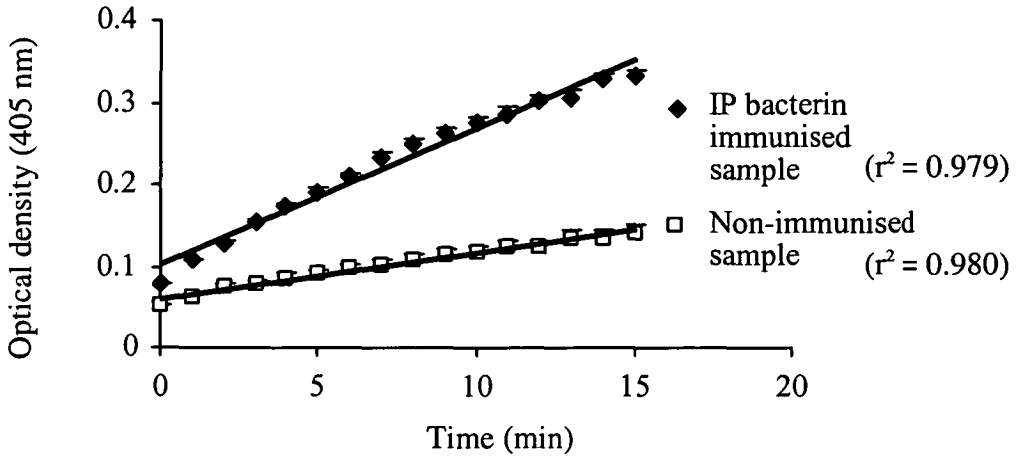

Figure 3.3 Colour development and subsequent optical density at 405 nm over an incubation period of 15 min in an ELISA to quantify anti-*V. harveyi* activity in barramundi serum. A linear relationship is indicated over this period. Immunised and non-immunised sera samples were run in duplicate. Values are means + SE.

3.5.5 Bacteriostatic assay

The bacteriostatic assay was modified from that of Thompson *et al.*, (1994). In this case, *V harveyi* suspension was grown to a density of approximately 5 x 10^8 cell mL^{-1}. Bacterial culture was initiated by inoculating 120 mL of NB2 with a cryopreservation bead (stored at -80°C) of the bacterium, grown overnight then inoculated into fresh media (3 mL). After incubation at 30°C in a shaking water bath for 2-2.5 h the cultures were in logarithmic growth phase. Cell densities were approximated from a standard curve constructed from a plot of $\log_{10}$ of cell densities versus absorbance at 550 nm measured during the growth phase of the bacteria. Seventy five microlitres of this culture was incubated with 25 µL of sample sera in 96 well flat bottom microtiter plates at 24°C for 1 h after which 100 µL of 3-(4, 5-dimethylthiozol-2-yl)-2, 5-diphenyltetrazolium bromide; thiazol blue (MTT- 5 mg mL^{-1} H_2O) diluted 1:10 in NB2 was added to each well, OD was immediately read at 550 nm and again after 15 min in the dark at room temperature with shaking. Differences in OD of samples and pooled non-immune standard barramundi sera standard (ΔOD) were calculated and antibacterial activity was determined by the formula :

[(ΔOD non-immune standard - ΔOD sample) /ΔOD non-immune standard] x 100

Results are expressed as a percentage inhibition of *V. harveyi* relative to the non-immune pooled standard sera. Samples were assayed in triplicate and all plates had pooled immune (I.S.) and non-immune sera standards (N.I.S.). In the standardisation of this assay, both immune and non-immune controls were tested for differences due to heat treatment and consequent complement inactivation. The standards were heated in a water bath to 47°C for 25 min, with temperature and time based on ranges for these parameters that inactivate complement in a number of teleosts as summarised by Sakai (1992). Attempts to add exogenous barramundi complement to the assay system were not possible due to lack of barramundi to supply sufficient quantities of serum. Fish complement is hemolytic as long as fish antibodies are used to sensitise red blood cells (Sakai, 1992), this suggests that complement from one fish species may be activated by antibody, in complex with antigen, from another species via the classical pathway. Therefore, as a substitute, serum from

southern bluefin tuna, *T. maccoyii*, with known complement (C) activity (M. Watts pers comm) was added to wells containing I.S. or N.I.S. in 80 µL volumes, after initial 1 h incubation period then incubated for a further 30 min before addition of MTT and absorbance measurements taken. Both heat-treated (complement inactivated) and non heat-treated standards were assayed in triplicate and control wells included those with tuna sera in lieu of standard sera and standard immune sera with heat-treated tuna sera. No enhancement of inhibitory activity was observed (Table 3.1). In some cases it was possible to have negative inhibition i.e. there was apparent growth of bacteria using serum as a nutrient, where this occurred the results are expressed as zero inhibition.

Table 3.1 *V. harveyi* inhibitory activity of immune (I.S.) and non-immune sera standards (N.I.S.) both heat-treated (complement inactivated) and untreated and after the addition of tuna sera as a complement (C) source. Values are means ± SE, n = 3.

Untreated Sera Sample	Inhibitory Activity (%)	Heat-treated Sera Sample	Inhibitory Activity (%)
I.S. + C-source	48.9 ± 5.5	I.S. + C-source	51.4 ± 2.5
N.I.S. + C-source	0*	N.I.S. + C-source	-5.0 ± 2.4 (0)
Tuna sera + C-source	-6.3 ± 1.9 (0)	Tune sera + C-source	-10.0 ± 2.7 (0)

* Used as non-immune standard to determine % inhibitory activity

To ensure the specificity of the assay for *V. harveyi* pooled non-immune and pooled immune sera were tested for inhibitory activity against *Vibrio anguillarum* and *Yersinia ruckeri*. In this case all three bacteria were cultured in NB2 broth (NaCl content was not increased to 1.5% for *Y. ruckeri*) to densities of approximately 10^8 cells mL^{-1} and the assay performed in triplicate as described above. The wells containing *Y. ruckeri* required incubation for 45 min

after the addition of MTT to note a substantial difference in OD. Neither non-immune nor immune sera displayed any real inhibitory activity toward *V. anguillarum* or *Y. ruckeri* (Table 3.2) whereas the immune sera had substantial inhibitory activity against *V. harveyi* (73.7%). The inhibitory activity of the pooled immune standard against *V. harveyi* in the specificity assay was higher than that observed for immune sera in the assay assessing the effect of complement inactivation (Table 3.1). This discrepancy was probably because the pooled immune sera in each assay were not identical and *V. harveyi* cell numbers were lower in the specificity assay.

For mucus samples, standard non-immune barramundi sera and the antiprotease buffer in which mucus samples were stored were also included on each plate. Mucus samples were dialysed for at least 72 h against PBS to remove antibacterial components of the antiprotease buffer. Additional controls run at the outset of the experiment but not on every plate included bacteria and MTT only, medium and MTT only and serum and MTT only.

Table 3.2 Inhibitory activity of pooled immune sera (I.S.) and pooled non-immune sera (N.I.S.) against three bacteria showing the specificity of anti-*V. harveyi* antisera for *V. harveyi.* Values are means ± SE, n = 3.

Sera sample	Inhibitory activity (%)		
	Vibrio harveyi	*Vibrio anguillarum*	*Yersinia ruckeri*
I.S.	73.7 ± 0.4	-2.9 ± 4.9 (0)	0.1 ± 3.5
N.I.S.	0*	0*	0*
Control (no serum)	0	0	0

* Used as non-immune standard to determine % inhibitory activity

CHAPTER 4

HUMORAL IMMUNE RESPONSE IN THE SERUM AND MUCUS OF BARRAMUNDI AFTER VACCINATION WITH FORMALIN-KILLED *Vibrio harveyi* CELLS VIA IMMERSION AND INTRAPERITONEAL INJECTION AND ASSESSMENT OF BACTERIOSTATIC ACTIVITY

4.1 INTRODUCTION

It seems accepted that delivery of vaccine by direct immersion falls somewhere between intraperitoneal injection and oral administration methods in terms of strength and consistency of protection (Palm *et al.*, 1998). Immersion vaccination is, however a commonly used administration method in fish culture for protection against bacterial pathogens like *V. anguillarum* (Smith, 1988) and *Y. ruckeri* (Ellis, 1988a). It is less stressful and more economical than individual application of a dose and in some cases has proven to be effective, even in the absence of measurable amounts of specific systemic antibody (Croy and Amend, 1977). This point is interesting because the protective role of antibody has been demonstrated after injection of *V. anguillarum* bacterin (Thuvander *et al.*, 1987) and passive immunisation (Harrell *et al.*, 1975). Effectiveness in the absence of specific serum antibody suggests that in some cases at least immersion vaccination seems to elicit some form of protection other than that of systemic antibody. Whether this equates to local or mucosal antibody or the cell-mediated immunity is still not known, although it is well established that antibody can be elicited in the mucus (refer Chapter 1).

An interesting observation is that vaccines delivered by IP injection are the most successful in terms of antibody response and protection even though in mammals at least vaccines delivered by injection are poor stimulators of mucosal immunity (Brown *et al.*, 1993; Hathaway and Kraehenbuhl, 2000). However, mucosally delivered antigens can stimulate

systemic antibody. Therefore protection after injection would be at the systemic level whereas protection after immersion delivery may well be at the mucosal level. This would partly explain why immersion is successful even in the absence of systemic antibody. It could also explain differing results from vaccination/ challenge trials where methods of challenge vary between immersion, co-habitation and direct injection. Since in fish most, if not all, pathogen entry is via the mucosa it seems reasonable to attempt to stimulate this immune compartment as an enhancement of the first line of defence. This presumes that fish actually possess a separate mucosal immune compartment and there is compelling evidence suggesting that this is the case (refer Chapter 1).

Typically, vaccines against bacterial pathogens are inactivated cultures of the particular bacterium. These are relatively simple to produce and are often very effective, though there are some notable exceptions (refer Chapter 1). Thus, the aim of this chapter was to investigate the immunogenicity in barramundi of a bacterin produced from a formalin-killed *V. harveyi* suspension in order to ascertain its potential as a prototype vaccine. The study aimed to quantify the level of antibody elicited in the serum and mucus of barramundi after immersion and intraperitoneal injection with the bacterin and to assess any resultant antibacterial activity.

4.2 MATERIALS AND METHODS

4.2.1 Fish Husbandry

Barramundi were procured and housed as described in section 3.2. Fish were randomly distributed into eight aquaria (12 fish per tank) using a lottery method and acclimated over 6 weeks. Water quality was monitored every 3-4 days and tanks were cleaned as required with 50% water exchanges occurring at least every week. Total ammonia levels never exceeded 0.25 mg L^{-1} and if nitrite levels exceeded 2.0 mg L^{-1} water exchanges were carried out. Before immunisations duplicate aquaria were randomly allocated a treatment using a lottery

method, with duplicate tanks for each immunisation method. The fish were weighed at commencement of the trial and the mean weight of fish in each treatment group appears in Table 4.1.

Table 4.1 Mean weight (± SE; n = 12) of barramundi per aquaria and treatment group at the commencement of the immunisation experiment using *V. harveyi* bacterin or saline delivered by intraperitoneal injection (IP) or immersion (IMM)

Treatment group		Replicate aquaria	
		1	2
Bacterin	IP	72.6 ± 2.2	73.0 ± 3.7
	IMM	72.5 ± 5.4	74.8 ± 3.1
Saline	IP	77.2 ± 3.0	70.8 ± 4.8
	IMM	88.4 ± 6.5	78.1 ± 5.0

4.2.2 Bacterin Preparation and Administration

A formalin-killed broth culture of *Vibrio harveyi* (strain no. 9056681) was used as a bacterin and was prepared as described in section 3.3. In this experiment the bacterin was administered to barramundi via immersion and intraperitoneal injection as described in section 3.3, however for intraperitoneal injection the bacterin was administered with an equal volume of Maintained® ISA-50 adjuvant. Twenty eight days after the primary immunisations, the fish were given an identical secondary dose or booster.

4.2.3 Sampling Procedures

At the time of booster immunisation blood samples were taken from individual fish via caudal puncture Fourteen days later all fish were lethally anaesthetised and blood, skin

mucus and gut mucus samples were taken as outlined in section 3.4. In this experiment skin mucus was sampled by gently scrapping the skin from the operculum to the caudal peduncle with the back of a scalpel blade and immediately placed in 1.5 mL tubes containing 0.5 mL of borate buffer with protease inhibitors (section 3.4) on ice. Blood was collected by caudal puncture or after severing the tail, allowed to clot at room temperature for several hours then kept at 4°C until serum was drawn off and stored at -20°C until assayed.

4.2.4 SDS-PAGE and immunoblotting

Western blots were performed to detect Ig in mucus samples. These samples were prepared by lyophilising for 48 h, reconstituing in 400 µL and 200 µL of sterile distilled water for the gut and skin mucus samples respectively and finally by centrifugation at 8800 x *g* for 5 min in a microcentifuge. Supernatants were heated (100°C for 4 min) in reducing buffer as previously described in section 2.2.2 and electrophoresed on 10% polyacrylamide gels at 200 v and 60 mA for 1 hour. Proteins were then electrotransferred from the polyacrylamide gels to nitrocellulose membranse as described in section 2.2.4 at 60 V and 200 mA for 55 min. Standard barramundi anti-*V. harveyi* (strain no. 9056681) serum was included as a control on all gels. Membranes were then probed following the protocol previously described (section 2.2.5) using rabbit anti-barramundi Ig (HC2) diluted 1:100 in 1% skimmed milk as the primary antibody.

4.2.5 Enzyme-linked immunosorbent assay (ELISA)

A standard indirect ELISA protocol using buffers and equipment as described in section 2.2.7 was followed to detect specific antibody in barramundi serum and mucus samples. Optimal dilutions of coating antigen (*V. harveyi* killed-whole cells; strain no. 9056681) and rabbit anti-barramundi Ig antiserum as secondary antibody (SpA-purified MBP 3) were determined firstly by doing a typical chessboard titrations (Crowther, 1995). Rabbit anti-barramundi Ig antiserum (starting at 1:200) was titrated against barramundi Ig (SpA-purified, starting at 10 µg mL^{-1} protein). The SpA-purified MBP 3 anti-barramundi Ig antiserum was used as it was the most appropriate in terms of titer and specificity (antiserum

designated HC 2 had not been produced at this time). Once a suitable working dilution (1:400) was determined for the rabbit anti-barramundi Ig antiserum, standard barramundi anti-*V.harveyi* antiserum (starting at 1:10) was titrated against whole *V. harveyi* cells (strain no. 9056681; starting at 220 μg bacterial cells mL^{-1} wet weight) in a second chessboard titration. Optimal antigen concentration of 18 μg mL^{-1} was chosen and this was used for subsequent processing of samples from this experiment. Optimal antigen concentration is defined as the least amount giving a significant OD reading (Arkoosh and Kaattari, 1992), in this case the highest OD within the linear portion of the titration curve. The standard anti-*V. harveyi* antiserum (immune standard) was titrated in duplicate against bacterial cell antigen to calculate a serum volume equating to 1 unit of antibody activity. Specific antibody activities of samples, expressed as units of antibody activity per volume of serum, were determined relative to the positive standard included on all plates and calculated as follows:

activity of sample = activity of standard x 50% volume of standard/ 50% volume of sample

where activity of standard is the reciprocal volume of the standard anti-*V harveyi* antiserum which gives 50% maximum OD, as determined from a titration curve, and is assumed to contain 1 unit of activity (Arkoosh and Kaatari, 1990). The activity of the standard is a constant in the equation, determined from its full titration, whereas the other terms are derived from individual plates and thus account for variations between plates. All samples and standards, both immune and non-immune, were titrated in duplicate from dilutions of 1:100 to 1:1600. Samples which displayed a maximum OD that was less than 50% of the maximum OD of the immune standard were designated as having no anti-*V. harveyi* activity.

4.2.6 Bacteriostatic assay

Bacterial inhibitory activity of sera and mucus were assayed as described in section 3.5.5.

4.2.7 Statistical analyses

Data were analysed with a one-way analysis of variance (ANOVA) using JMP 3.2.1 statistical package. ANOVA assumptions of data normality and homogeneity of variance were tested using the Shapiro-Wilk W test and Bartlett's test respectively. Nested analyses were used to find differences in replicate aquaria within treatment groups for specific anti-*V. harveyi* antibody activity and bacterial inhibitory activity. Differences were highlighted as a multiple comparison of means was carried out (Tukey-Kramer HSD test). Serum *V. harveyi* bacterial inhibitory data failed to fulfill assumptions of normality and homogeneity of variances both before and after common logarithmic transformation, therefore a non-parametric test (Kruskal-Wallis test) was used for statistical analysis. Antibody activities within treatment groups after primary and secondary immunisations were compared using a t test for paired comparisons. A common logarithmic transformation was applied to these data to comply with the data normality and homogeneity of variance assumptions. Correlation coefficients were determined and tested for significance (Sokal and Rohlf, 1987) when looking at associations between specific antibody activity and bacterial inhibitory activity. For all tests a significance level of $P < 0.05$ was adopted.

4.3 RESULTS

4.3.1 SDS-PAGE and immunoblotting

Electrophoretic profiles of the skin and gut mucus revealed a number of bands within the 97 and 31 kDa size range (Figure 4.1) however, western blotting detected no strong bands of corresponding MW to either LC or HC Ig in any mucus samples that were representative of all immunised groups of fish (Figure 4.2). Anti-barramundi LC and HC Ig antisera both showed bands corresponding to LC and HC components in the control serum but only HC 2 showed faint bands in mucus samples corresponding to the MW of HC Ig (Figure 4.2A).

4.3.2 ELISA optimisation

The optimal dilution of the rabbit anti-barramundi antiserum (MBP 3) chosen was 1:400 as determined from the chequerboard titration of the antiserum against the protein A-purified barramundi Ig (Figure 4.3). A strong signal (OD 492 nm of 1.5) is recorded at this dilution, although a 1:200 did increase the OD and was sufficient to eliminate background staining in a Western blot (see section 2.3.3), 1:400 was considered more appropriate for the more sensitive ELISA. The chequerboard titration of barramundi anti-*V. harveyi* standard antiserum against whole *V. harveyi* cells (Figure 4.4) indicated that optimal *V. harveyi* antigen concentration was approximately 18 $\mu g\ mL^{-1}$ wet weight. This concentration of antigen gave OD readings greater than 1.5 at all the antisera dilutions. The titration of the standard anti-*V. harveyi* antiserum and subsequent regression of the linear portion of the curve (Figure 4.5A & B) showed that 50% of the maximum OD of the standard was 0.520. The volume of the serum standard giving this OD was calculated as 4.1×10^{-3} µL from the equation of the straight line portion of the curve. Therefore the activity of the serum standard is 243 units μL^{-1} .

4.3.4 Specific anti-*V. harveyi* antibody activity and bacterial inhibitory activity

Specific antibody activity was detected in both IP and IMM bacterin-immunised groups of barramundi after both primary and secondary immunisations (Figures 4.6 & 4.7). There was some activity noted in control groups 28 days after primary immunisations (Figure 4.6) but this was limited to 4 individuals. No differences were detected between aquaria within treatments after either primary or secondary treatments ($P = 0.35$ & $P = 0.25$), thus the data were pooled at this level. The antibody response was significantly higher in the IP-treated group after each immunisation ($P = 0.01$ & $P < 0.0001$) and possible evidence of an anamnestic response was seen as significantly higher levels of antibody activity were recorded after the secondary immunisation ($P = 0.0025$) (Figure 4.8). There was no concomitant increase in antibody activity noted in the IMM-treated group. The ELISA found no anti-*V.harveyi* activity in any of the mucus samples.

As with the specific antibody levels serum bacteriostatic activity was significantly higher in IP-bacterin treated fish ($P < 0.001$) (Figure 4.9). There was also activity noted in sera from 5 individuals from control groups, although these were not the same fish that demonstrated antibody activity. Examination of the relationship between serum antibody levels and bacteriostatic activity revealed no statistical correlation between these parameters (Figures 4.10 & 4.11).

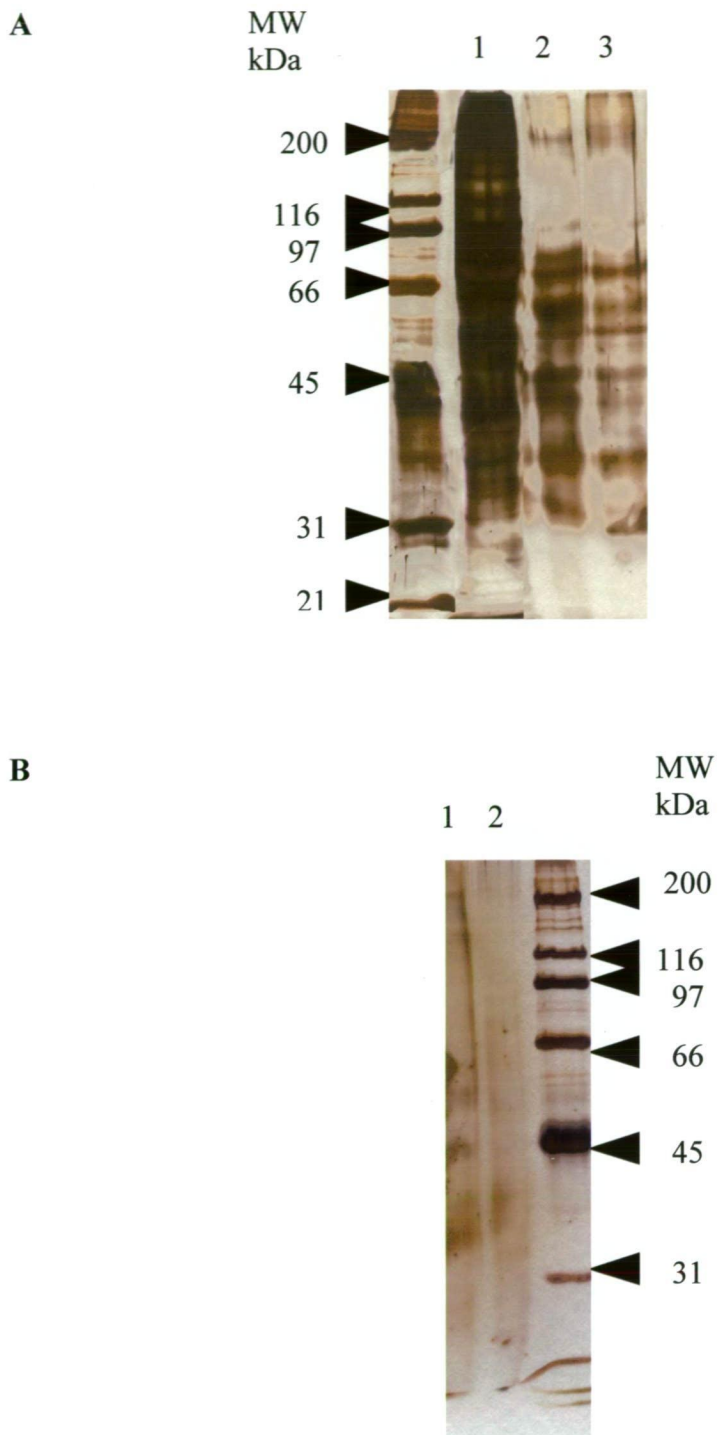

Figure 4.1 Electrophoretic profiles of skin mucus and gut washings from barramundi subjected to SDS-PAGE in 10% gel under reducing conditions. **A**. Barramundi serum (lane 1) and skin mucus (lanes 2 & 3). **B**. Gut washings (lanes 1 & 2). Both gels were silver stained.

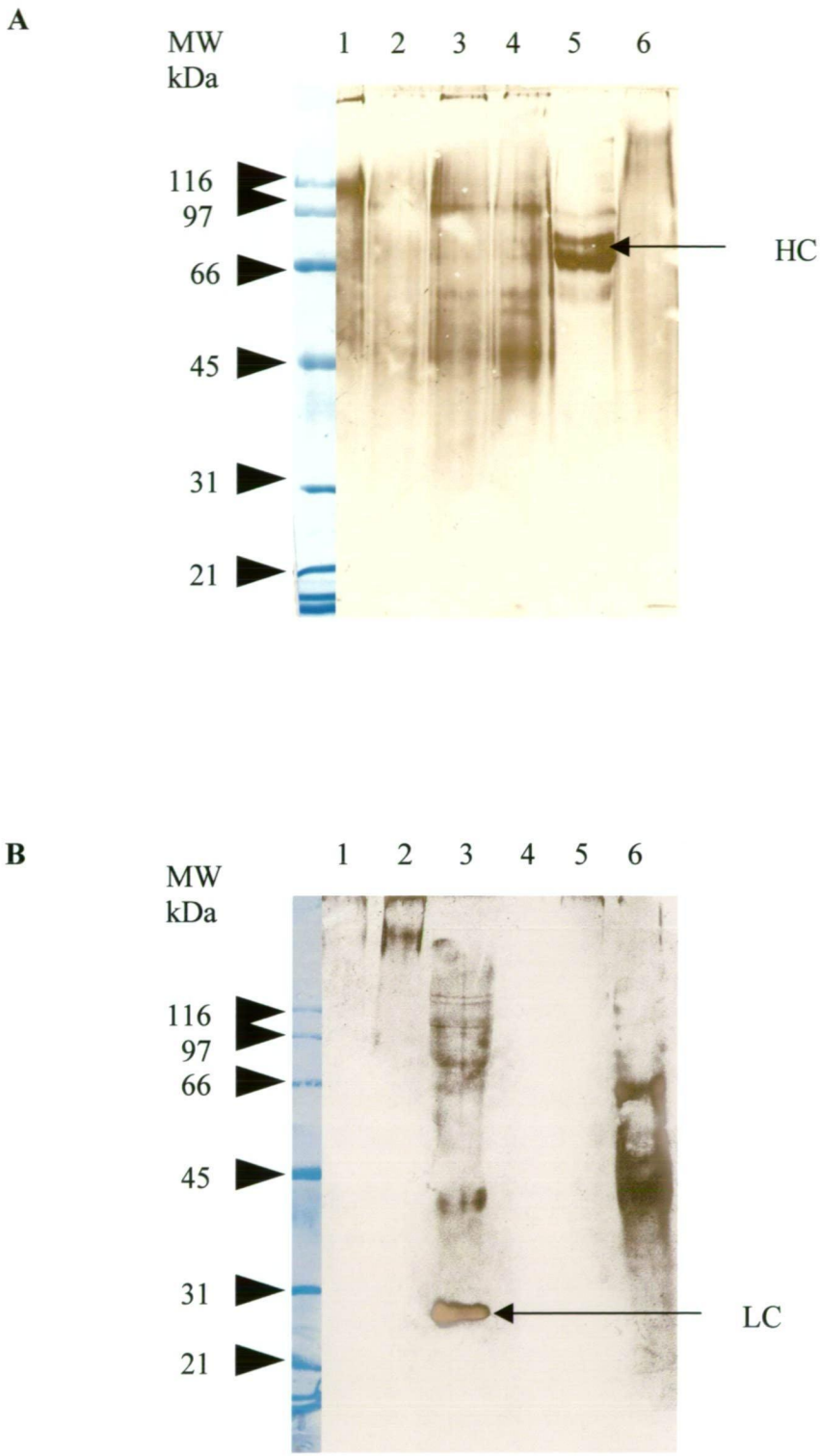

Figure 4.2 Immunoblots of skin and gut mucus from barramundi . **A**. Probed with HC 2 at 1:100; skin mucus (lanes 1-4), barramundi serum with heavy chain Ig (HC) labelled (lane 5) and gut mucus (lane 6) **B.** Probed with LC 1 at 1:100; gut mucus (lanes 1 & 2), barramundi serum with light chain Ig (LC) labelled (lane 3) and skin mucus (lanes 4-6).

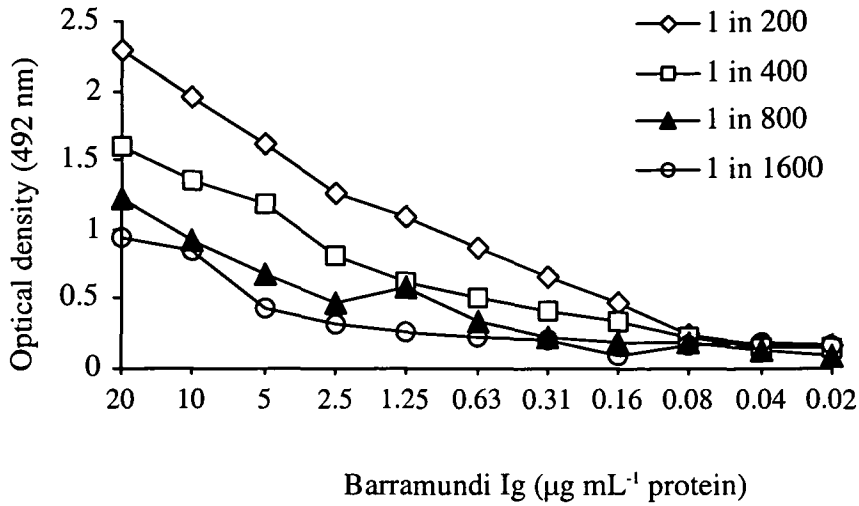

Figure 4.3 Chequerboard titration of rabbit anti-barramundi Ig antiserum (SpA-purified MBP 3) serially diluted from 1:200 to 1:1600 against SpA-purified barramundi Ig serially diluted from 20 to 0.02 μg mL^{-1} protein.

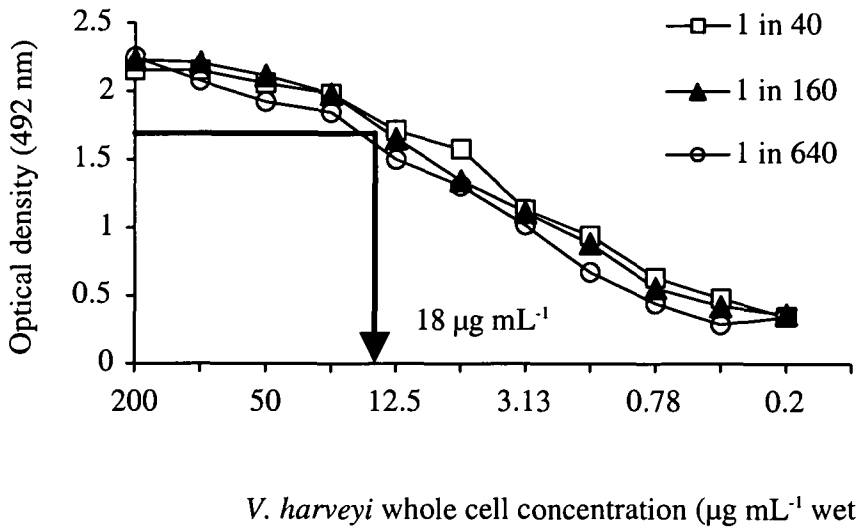

Figure 4.4 Titration of *V. harveyi* whole cell antigen against various dilutions of barramundi anti-*V. harveyi* standard antiserum indicating an optimal antigen concentration of 18 μg mL^{-1} (wet weight).

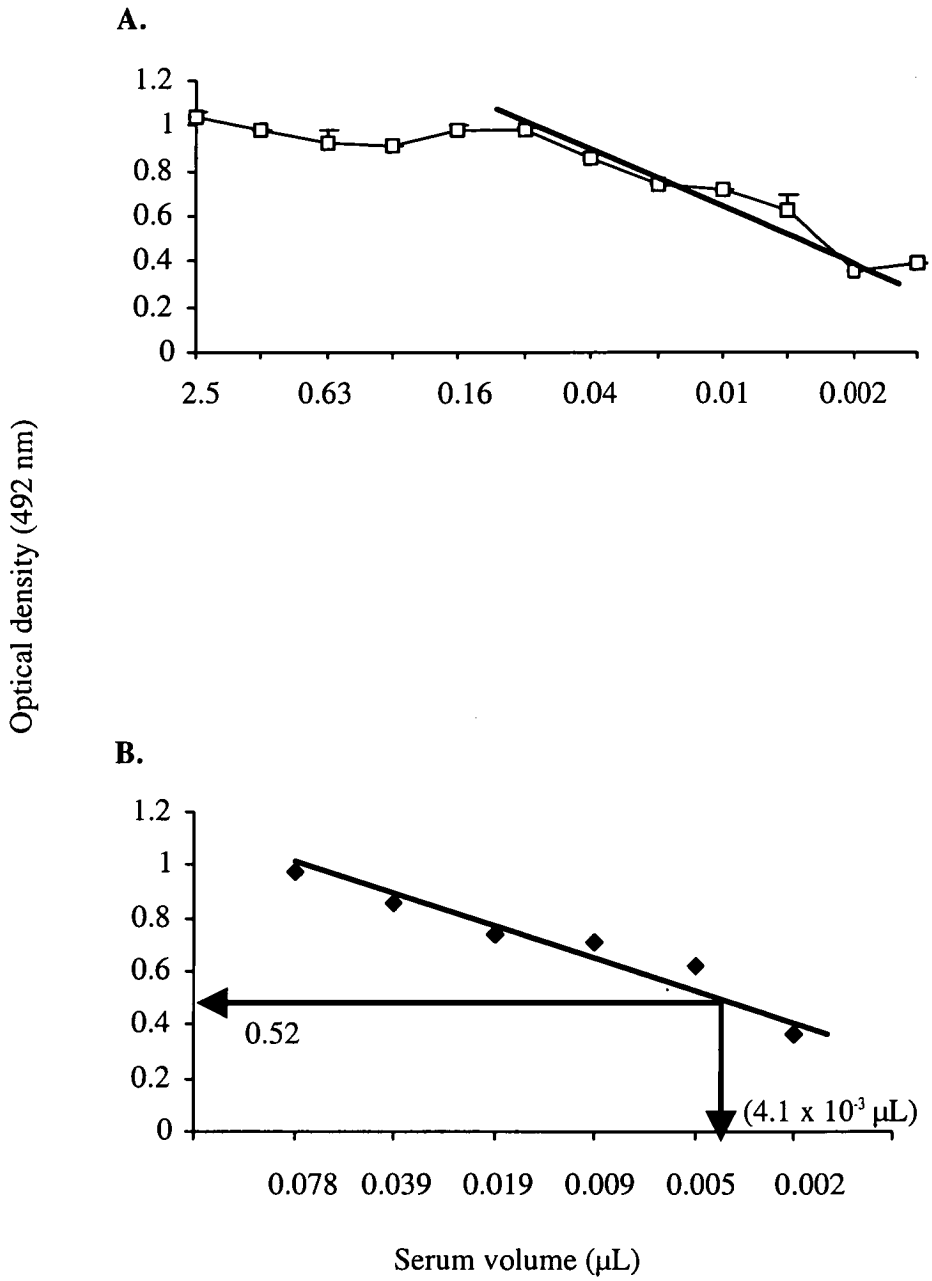

Figure 4.5 Titration curve and linear regression of barramundi anti-*V. harveyi* (strain no. 9056681) antiserum. **A.** Antiserum titrated through dilution series of 1:20 to 1:40960 against *V. harveyi* (strain no. 9056681) whole cell antigen coated at 18 µg mL^{-1} (wet weight). **B.** Regression of linear portion of curve (r^2 = 0.929) showing the volume of serum giving 50% of the maximum OD from which a unit of antibody activity is derived.

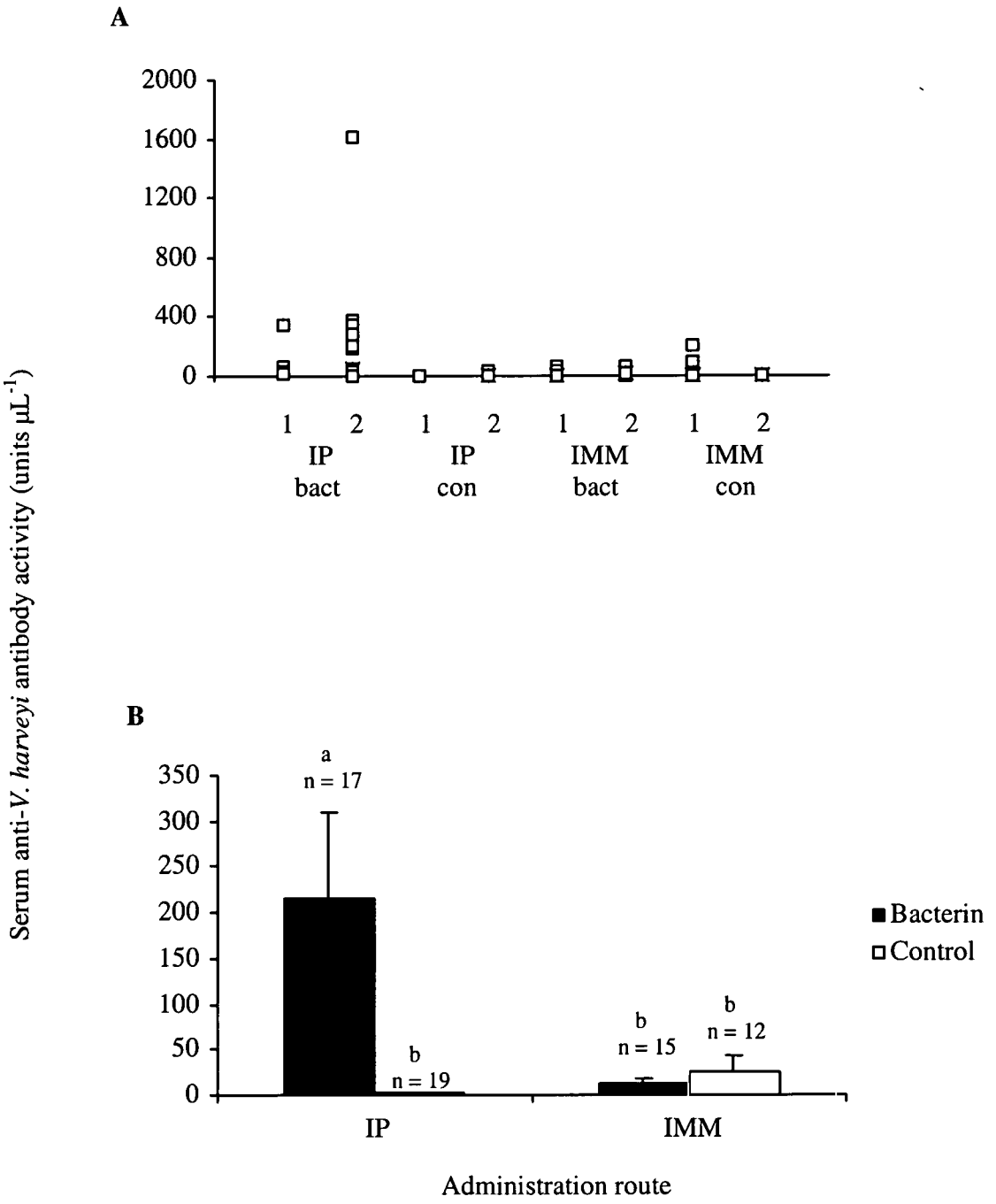

Figure 4.6 Specific anti-*V. harveyi* activity of barramundi serum 28 days after primary immunisation with *V. harveyi* bacterin or saline via intraperitoneal injection (IP) with Montanide® ISA-50 adjuvant, or immersion (IMM). **A.** Individual fish within 2 aquaria per treatment group. **B.** Fish per treatment group, values are means + SE and disparate superscripts show significant differences (P = 0.01, Tukey-Kramer HSD test).

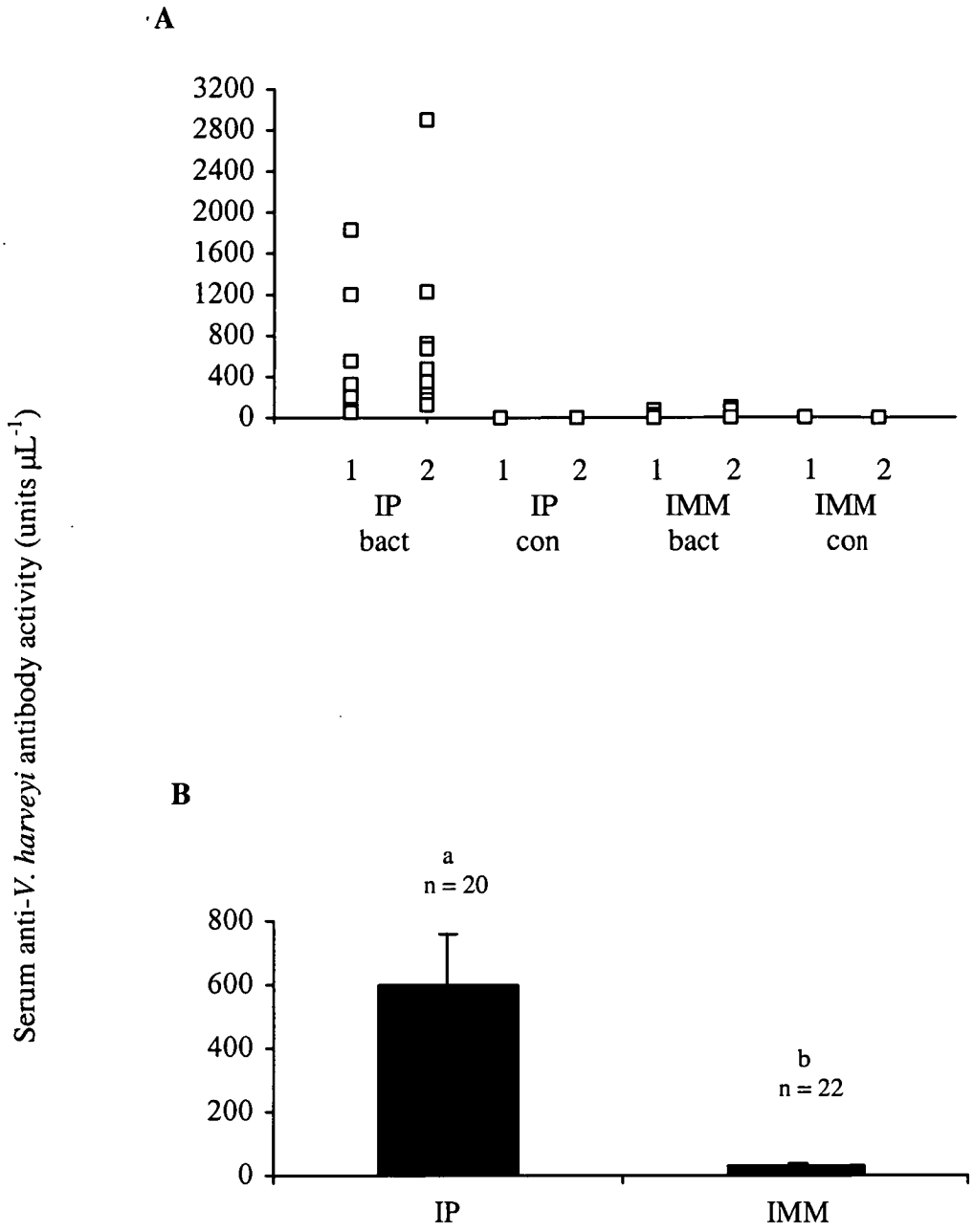

Figure 4.7 Specific anti-*V. harveyi* activity of barramundi serum 14 days after secondary immunisation with *V. harveyi* bacterin via intraperoitoneal injection (IP) with Montanide® ISA-50 adjuvant, or immersion (IMM). **A.** Individual fish within 2 aquaria per treatment group. **B.** Fish per treatment group, values are means + SE and saline treated controls showed no antibody activity. Disparate superscripts show significant differences ($P < 0.0001$, Tukey-Kramer HSD test).

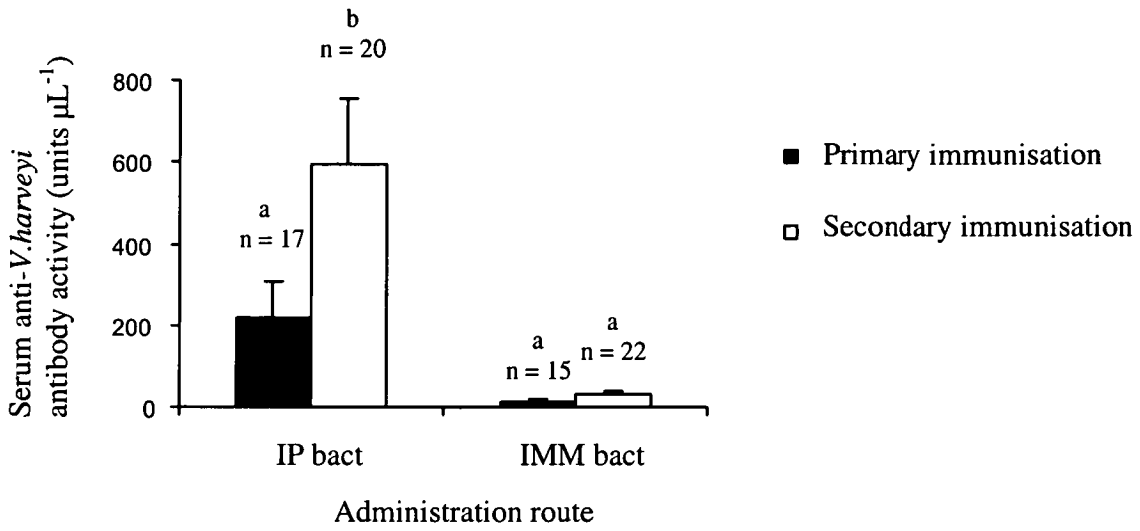

Figure 4.8 Specific anti-*V. harveyi* antibody activity of barramundi serum immunised with *V. harveyi* bacterin (bact) via intraperitoneal injection (IP) with Montanide® ISA-50 adjuvant, or immersion (IMM) after primary and secondary immunisations. Values are means + SE, and disparate superscripts significant differences within a treatment (P = 0.003, paired t Test).

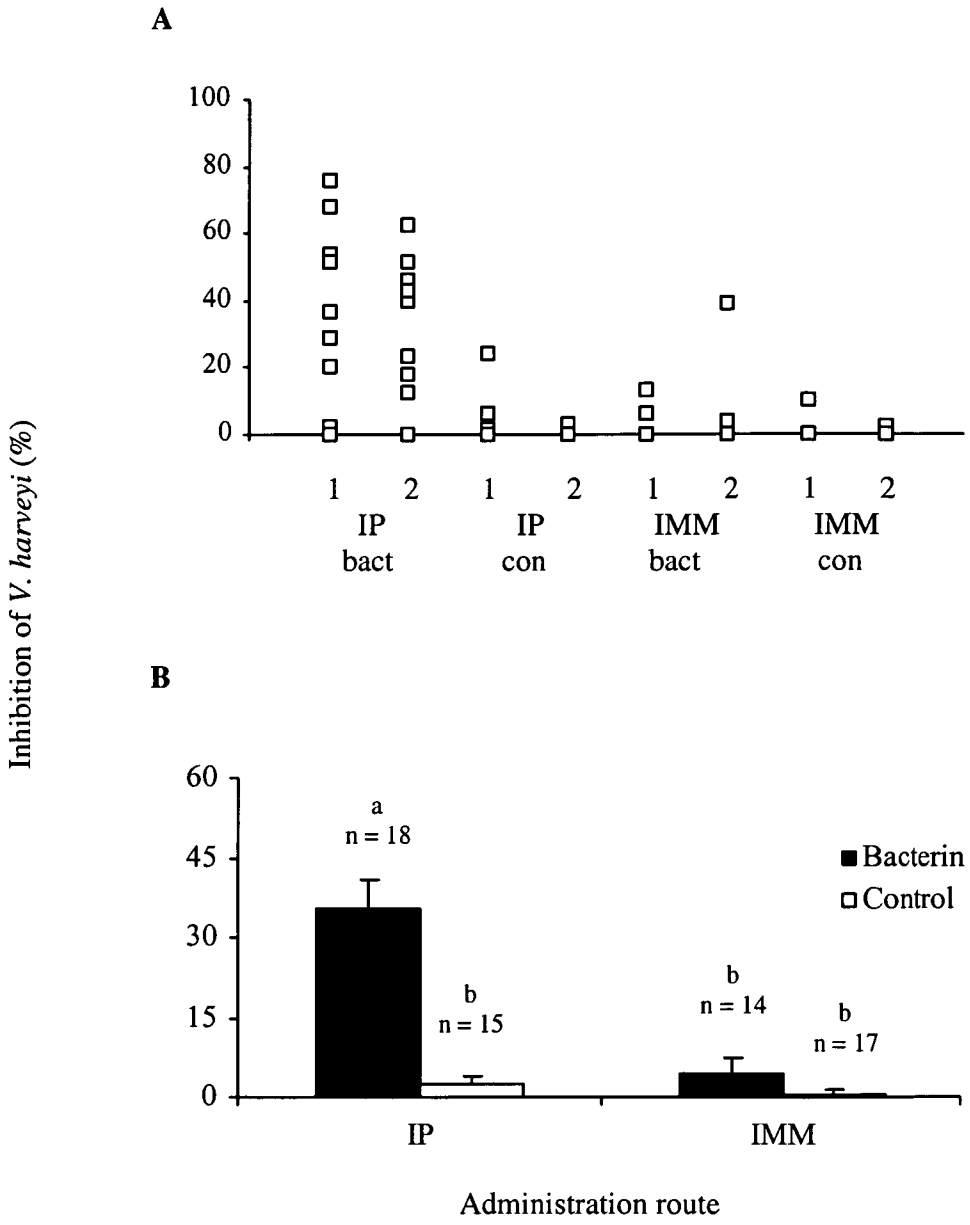

Figure 4.9 Serum bacteriostatic activity of barramundi after immunisation with *V. harveyi* bacterin via intraperitoneal injection (IP) with Montanide® ISA-50 adjuvant and immersion (IMM) 14 days after secondary immunisations. **A.** Individual fish within 2 aquaria per treatment group of bacterin (bact) or saline control (con). **B.** Activity of fish sera from bacterin treated groups only, values are means + SE and disparate superscripts show significant differences ($P < 0.001$, Kruskal-Wallis Test)

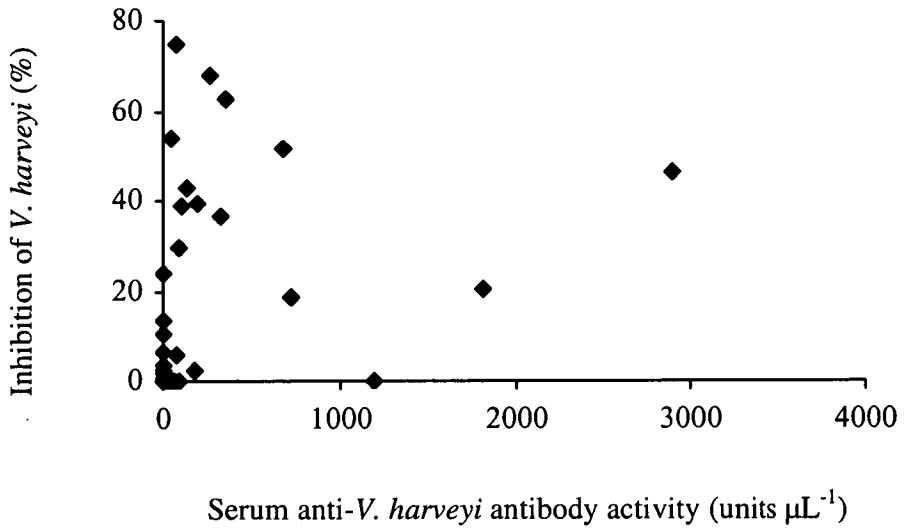

Figure 4.10 Relationship between anti-*V. harveyi* antibody activity and bacteriostatic activity of serum from barramundi immunised with *V. harveyi* bacterin administered by intraperitoneal injection with Montanide® ISA-50 adjuvant or immersion and including individuals from saline treated control groups displaying bacteriostatic and/or anti- *V. harveyi* antibody activity ($r = 0.234$, $n = 31$).

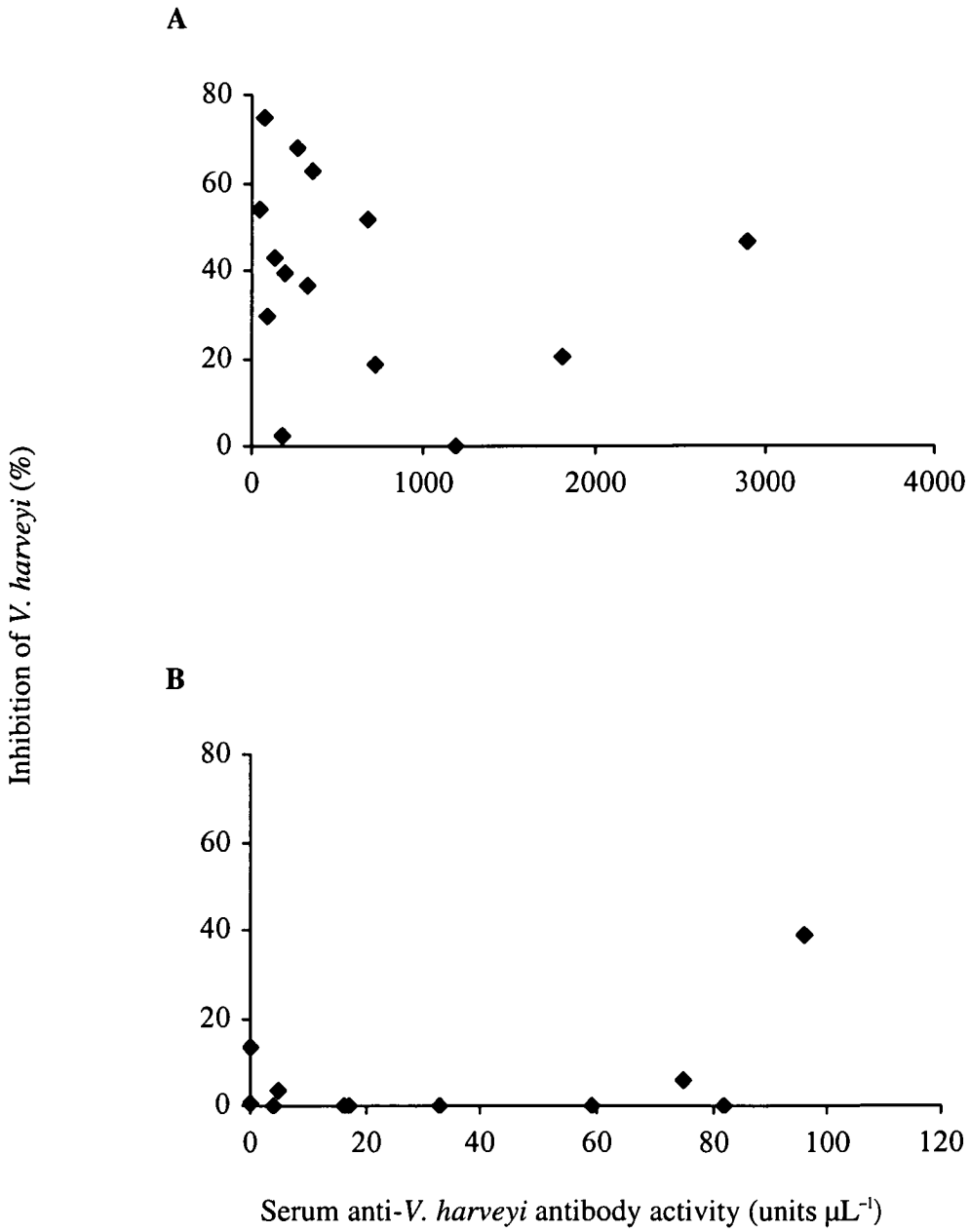

Figure 4.11 Relationship between barramundi serum anti-*V. harveyi* antibody activity and serum bacteriostatic activity showing no significant correlation ($P > 0.05$) when examined by individual treatment group. **A.** Bacterin administered by intraperitoneal injection with Montanide® ISA-50 adjuvant ($r = 0.218$, $n = 14$). **B.** Bacterin administered by immersion ($r = 0.472$, $n = 11$).

4.4 DISCUSSION

A formalin-killed *V. harveyi* whole cell preparation will be useful as a prototype vaccine for barramundi. All bacterin-immunised fish produced systemic antibody to *V. harveyi* whole cells with IP injected barramundi displaying substantially higher (a ten-fold increase) antigen-specific antibody activity compared to the IMM group. In addition, there was approximately a three-fold increase in antibody activity 14 days after a secondary IP injection. Both increases in specific antibody activity were statistically significant.

These findings raise two points. First, the comparatively lower humoral response after immersion bacterin delivery and, second the indication of an anamnestic response after IP injection but not immersion. The relatively lower response of the IMM group concurs with much of the literature describing little or no systemic humoral responses in fish after immersion immunisation (e.g. Baba *et al.*, 1988; Thorburn and Jansson, 1988; Nakanishi and Otoake, 1997) and when comparing responses to IP and IMM delivered antigen (e.g. Whittington *et al.*, 1994; Thune *et al.*, 1997). However, some studies report elicitation of specific antibody of a similar magnitude after secondary immunisations when comparing antigen administration methods (Palm *et al.*, 1998). Differences probably relate to the type of antigen and its ability to be transported and exposed to the systemic immune system. The magnitude of the immune response depends, among other factors, on antigen concentration and its physical nature i.e if it is particulate or soluble (Nakanishi and Ototake, 1997). During exposure by immersion the main route of antigen uptake is variously attributed to the skin (Ototake *et al.*, 1996), gills (Bowers and Alexander, 1981; Smith, 1982; Zapata *et al.*, 1987), and via the intestinal tract after ingestion (Robohm, 1986; Robohm and Koch, 1995). Tatner (1987) found significant amounts of radiolabeled *A. salmonicida* in the gut of rainbow trout administered by bath but could not conclude that that this site was totally responsible for the induced immunity. In the studies referred to above measuring responses after immersion and/or IP injection different antigens and fish species were used which could explain the disparity of the results. In the present experiment the lower response after immersion immunisation may have been due to less antigen uptake relative to IP injection

and thus a lower antibody response. Whether or not the differences observed in humoral responses after each administration method parallel differences in protection is not known given the lack of vaccination/challenge data. It is clear that immersion immunisation stimulates several immune mechanisms, both cell-mediated and humoral, which contribute to disease prevention (Nakanishi and Ototake., 1997) but many aspects of immersion immunisation remain controversial. For example, the type of immunity induced, the relationship between systemic antibody and protection and, the major route of antigen uptake after immersion antigen delivery are all uncertain.

Regarding the indication of an anamnestic response, in this study IP-injected fish received the bacterin with an oil-based adjuvant which may result in a magnified antibody response. In the context of the aim of the study an adjuvant was used in the IP administration to ensure a detectable response. However, observations of a heightened antibody response after secondary IP immunisation may be indicative of memory induction or, may equally be due to the adjuvant. The adjuvant used was Montanide® ISA-50 which is classified as an immunostimulatory oil emulsion (Leenaars *et al.*, 1999) and would have acted as an antigen reservoir and effectively prolonged retention of the antigen. Subsequent studies where bacterin is administered without adjuvant have been carried out and are reported later in this thesis.

The bacterial inhibitory activity of the serum from both bacterin-treated groups of fish highlights the potential of the bacterin as a vaccine. This activity was highest in IP-immunised fish which was six times greater than in IMM-immunised fish. Although there was no apparent correlation between antibody activity and bacteriostatic activity, the IP-treated group showed consistently higher levels for both activities.

The lack of detection of antigen-specific or any antibody in mucus samples from any treatment group by either ELISA or Western blot may mean that either there was no antibody in mucus samples, mucosal Ig is sufficiently different to be less reactive with anti-barramundi serum Ig reagents, or detection techniques used in this study are not sensitive enough. Lack of detectable Ig in the skin mucus was surprising given that Ig does exist in

fish mucus and has been shown to be inducible in cutaneous mucus of teleosts after immersion immunisation (Lobb, 1987), IP injection (Ourth, 1980; St. Louis-Cormier *et al.*, 1984) and enteric antigen delivery (Rombout *et al.*, 1986; Rombout *et al.*, 1989; Wong *et al.*, 1992; Jenkins *et al.*, 1994; Cain *et al.*, 2000). It is therefore unlikely that no Ig was present in the cutaneous mucus of barramundi. Though Rombout *et al.* (1993) demonstrated differences between mucus and serum Ig in carp, *C. carpio*, via differential recognition with mAbs, polyclonal antisera were used in this study. Such antisera would be expected to be reactive with both forms of Ig, given they are a heterogeneous mixture of antibodies reactive with numerous epitopes on an Ig molecule. It therefore seems likely that antibody was present in the skin mucus of barramundi but at levels too low to detect using current methods. Thus, it cannot be ascertained from this experiment whether or not immersion immunisation with a bacterin elicits an antibody response from the mucosa.

The apparent lack of Ig in the gut mucus may similarly indicate levels too low to detect or, that no antigen reached the gut-associated lymphoid tissue (GALT), which in teleosts are not of the organised nature as found in mammals (Press and Evensen, 1999). Though it is reasonable to assume that during immersion some antigen would be ingested and have an opportunity to reach the gut, Ototake *et al.* (1996) proposed that after immersion vaccination the skin is the predominant site of antigen uptake and in their study little or no antigen was found in the intestine. Therefore, in this study it is possible that no bacterin reached the intestine, at least in an immunogenic form. A number of studies have reported antigen uptake in the intestine (e.g. Rombout *et al.*, 1986; Joosten *et al.*, 1995; Joosten *et al.*, 1996) though in most cases antigen was delivered via the oral or anal route and not by immersion. Another possibility for the failure to detect Ig in the gut washings is that any antibody secreted into the gut mucus was transient. In mammals, the majority of locally produced and secreted Ig at the mucosal surfaces is IgA (McGhee *et al.*, 1992), an isotype resistant to proteolytic degradation. Fish lack secretory Ig analogous to IgA in mammals (Ellis, 1999) and consequently fish Ig may be subject to proteolytic attack on secretion into the gut environment.

CHAPTER 5

COMPARISON OF INTRAPERITONEAL INJECTION, ANAL INTUBATION AND IMMERSION AS BACTERIN ADMINISTRATION TECHNIQUES FOR BARRAMUNDI

5.1 INTRODUCTION

One area of research in fish vaccinology involves administration methods in an effort to identify those that are most economic, impose the least stress and stimulate the appropriate immune response. There are basically three methods of delivering antigens to fish to stimulate the immune systems and confer protection against infectious disease; by injection, immersion in an antigen suspension or, perorally. Each has advantages and disadvantages in terms of economics, stress and efficacy. Injection requires individual handling and anaesthesia and often the use of adjuvant, but delivers a precise amount of antigen and is usually the most effective (Ellis, 1988b) and, in some cases, is the only effective administration method e.g. for furunculosis when an adjuvant is required (Newman, 1993; Press and Lillehaug, 1995). Immersion is less stressful than injection as it requires no individual manipulation, however, it is less precise and may require greater volumes of vaccine when larger fish are being immunised. Immersion exposure to antigen does parallel the natural entry route of pathogens (Moore *et al.*, 1998), is practical for mass vaccination and, is currently the delivery method used for many commercial vaccines (Nakanishi and Ototake, 1997). Immersion vaccine delivery has a variable success rate for a number of bacterial diseases but it is quite effective against diseases caused by *V. anguillarum* and *V. ordalii* (Newman, 1993). Peroral antigen delivery, where vaccine is incorporated into food, is the least stressful, requires no individual handling or group moving but it is also the least successful to date compared to immersion and injection (Rombout *et al.*, 1986; Lillehaug, 1989b; Dec *et al.*, 1990) and, it is difficult to ensure that each individual receives an

effective dose. Nevertheless, peroral delivery is often seen as the most desirable means of vaccine delivery because of its advantages. Vaccination of fish by immersion and perorally have the potential to stimulate mucosal immunity which, as mentioned in Chapter 1, has some autonomous function and may be the preferred site of immune induction given that many pathogens initiate infections at these sites (McGhee *et al.*, 1992).

Desirability to develop oral vaccines has led to investigation of antigen uptake in the gut and exposure to cells of the gut-associated lymphoid tissue (GALT) and this is often achieved by peranal administration of antigen. The priority being to ascertain whether the antigen can be presented to lymphoid cells via the gut, then if successful, means of delivering the antigen to the gut perorally can be explored. The lack of success of oral vaccine delivery is often attributed to antigen degradation in the acidic environment of the stomach before it reaches responsive areas in the hind gut (Johnson and Amend, 1983; Rombout et al., 1986; Lillehaug, 1989a). Substantial efforts have been employed to find means of protecting antigens from gastric and pancreatic secretions, including various forms of encapsulation (Joosten, *et al.*, 1995; O'Donnell *et al.*, 1996; Azad *et al.*, 1999) and coating with pH sensitive polymers (Piganelli *et al.*, 1994). Results from these efforts have been promising in terms of antigen uptake, serum antibody titers and survival after challenge.

This study attempted to compare immune responses in barramundi to *V. harveyi* bacterin administered via various routes. The immune systems of barramundi have not been extensively studied, therefore information on how this species responds to different vaccine administration techniques will be valuable. To that end the aim of this experiment was to investigate both the specific humoral response and non-specific immune parameters after administration of a *V. harveyi* bacterin via intraperitoneal injection, immersion and anal intubation, the latter method to ensure vaccine delivery to the gut-associated lymphoid tissue. A priority was to confirm the ability of barramundi to mount a systemic and/or mucosal antibody response after peranal bacterin delivery. This study differs from the previous one described in Chapter 4 in that no adjuvant was used for any bacterin administration method and a more pathogenic strain of the bacterium was used. This strain was used as it has been identified as more pathogenic subsequent to the experiment detailed

in Chapter 4 and because the strain was to be used as a prototype vaccine in commercial trials.

5.2 MATERIALS AND METHODS

5.2.1 Fish Husbandry

Barramundi were procured and housed as described previously in section 3.2 and were acclimated over 4 weeks. Due to the large numbers of aquaria used for this trial commercial heaters were installed to maintain uniform temperatures in all aquaria (mean temperature was 26.5°C ± 0.3). Prior to commencement of the trial duplicate aquaria, each containing 10 fish, were randomly allocated a treatment using a lottery method and fish were similarly randomly allocated to aquaria. The fish were weighed at the commencement of the trial and the mean weight of fish in each treatment group appears in Table 5.1.

5.2.2 Bacterin preparation and administration

The bacterin was prepared from *V. harveyi* strain no. 9050405 as previously described for strain no. 9056681 in section 3.3. Bacterin administration methods were intraperitoneal injection (IP), immersion (IMM) and anal intubation (AI) to facilitate delivery to the GALT and were carried out as described in section 3.3. Actual doses were 200 µL of vaccine (approximately 0.08 mg cells wet weight per gram of fish) for IP injection, a 60 s dip in 1:5 dilution of the bacterin containing approximately 2 x 10^7 cells mL^{-1} for IMM and, 300 µL of vaccine (approximately 0.12 mg cells wet weight per gram of fish) for AI. Placebos, consisting of seawater for the immersion and saline with 0.5% formalin for the intubation and intraperitoneal injection, were administered to fish using the same methods. In addition to these treatment groups a control group remained naive to all treatments and handling. All fish were bled and re-immunised as above after 21 days.

Table 5.1 Mean weight (± SE; n = 10) of barramundi per aquaria and treatment group at the commencement of the immunisation experiment using *V. harveyi* bacterin or saline delivered by intraperitoneal injection (IP), immersion (IMM) and anal intubation (AI)

Treatment group		Mean weight (g) in replicate aquaria	
		1	2
Bacterin	IP	98.3 ± 7.6	104.5 ± 5.9
	IMM	89.1 ± 4.2	88.3 ± 3.6
	AI	95.0 ± 4.9	98.9 ± 3.7
Saline	IP	95.6 ± 5.4	99.8 ± 4.1
	IMM	94.2 ± 3.0	92.9 ± 4.5
	AI	100.2 ± 8.3	83.3 ± 2.9

5.2.3 Sampling Procedures

Prior to sampling all fish were starved for 48 h. Ten days post booster five fish from each aquarium were lethally anaesthetised, sampled for blood, mucus (skin and gut) and head kidney. Blood was collected by caudal puncture, allowed to clot then stored at 4°C overnight before serum was removed and frozen at -20°C. Skin mucus samples were collected using a cane skewer as described in section 3.4, similarly, gut mucus and head kidney tissue were collected as previously detailed in sections 3.4 and 3.5.1 respectively. The remaining five fish from each aquarium were similarly processed 21 days post booster. Sampling periods after booster of 10 and 21 days were chosen so as to reflect a reasonable time frame in which to detect differences in the parameters to be measured. The time frame was based on previous reports for detection of responses after booster vaccination (Ainsworth *et al.*, 1995; Joosten *et al.*, 1996; Palm *et al.*, 1998).

5.2.4 Immune assays

Macrophage isolation from head kidney tissue and estimation of phagocytic indices of this cell suspension were carried out according to procedures detailed in sections 3.5.1 and 3.5.2. Assays to measure serum lysozyme, anti-*V. harveyi* antibody activity by ELISA and bacteriostatic ability were performed on sera and mucus samples using protocols previously detailed (sections 3.5.3 - 3.5.5).

The ELISA was performed using sonicated *V. harveyi* (strain no. 9050405) cells as the coating antigen. This differs from whole cell coating of strain no. 9056681 used for ELISA described in Chapter 4 as whole cells of strain no. 9050405 failed to adhere uniformly to plates. An additional modification to the ELISA used in Chapter 4 was the use of a different anti-barramundi Ig antiserum (HC 2) as secondary antibody which was subsequently found to be more specific. Recalibration of the assay found an optimal working concentrations of 1:400 dilution for HC 2 and antigen coating concentration of 5.5 $\mu g\ mL^{-1}$ protein for the bacterial sonicate (Figure 5.1 & 5.2). The strain difference also required production of pooled barramundi anti-*V harveyi* antisera for use as a positive standard to be used on all plates. This was produced by immunising six fish by IP injection with 0.08 mg (wet weight) bacterin per gram of fish with an equal volume of Montanide® ISA-50 adjuvant, re-immunising after 4 weeks, then harvesting serum after a further 2 weeks. The sera were then pooled and titrated in duplicate against the bacterial sonicate (Figure 5.3A) and linear regression used to calculate the volume of sera equating to 1 unit of antibody activity. As detailed in section 4.2.5 the specific antibody activities of samples are expressed as units of antibody activity per volume of serum, although in this case the activity of the standard was the reciprocal volume of the pooled sera which yielded 40% of the maximum OD. The volume of sera giving 40% of the maximum OD was 8.3×10^{-3} µL therefore the activity of the sera standard is 120 units μL^{-1}. This differs to the 50% maximum OD used for calculations in the experiment reported in Chapter 4 because there was a greater difference between the activities of the samples and the standard antisera. All samples and standards were titrated from dilutions of 1:50 to 1:3200.

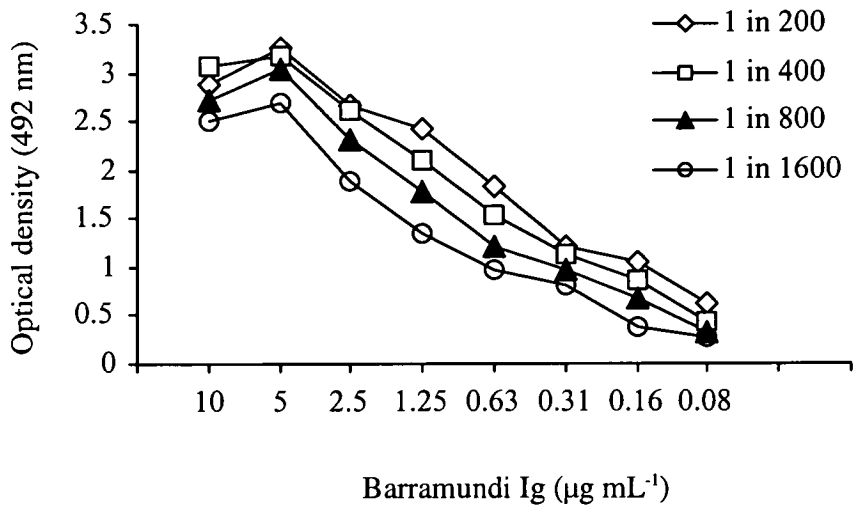

Figure 5.1 Chequerboard titration of rabbit anti-barramundi Ig antiserum (HC 2) serially diluted from 1:200 to 1:1600 against SpA-purified barramundi Ig serially diluted from 10 to 0.08 μg mL^{-1} protein.

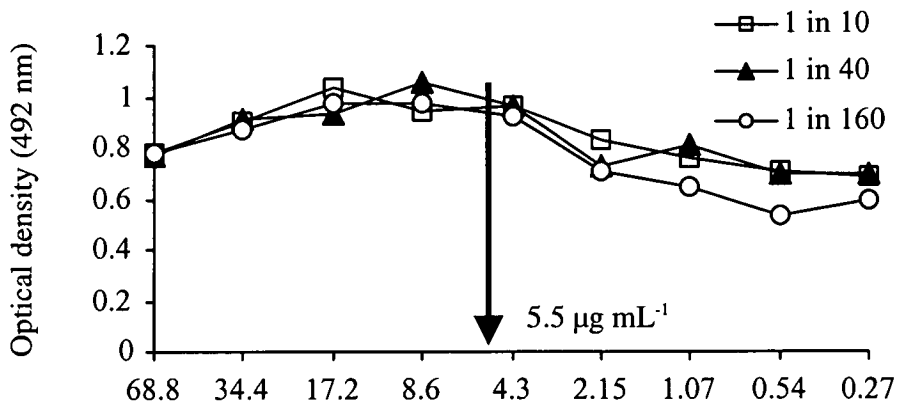

Figure 5.2 Titration of *V. harveyi* sonicate (strain no. 9050405) against various dilutions of barramundi anti-*V.harveyi* (strain no. 9050405) standard pooled antisera indicating an optimal protein concentration of approximately 5.5 μg mL^{-1}.

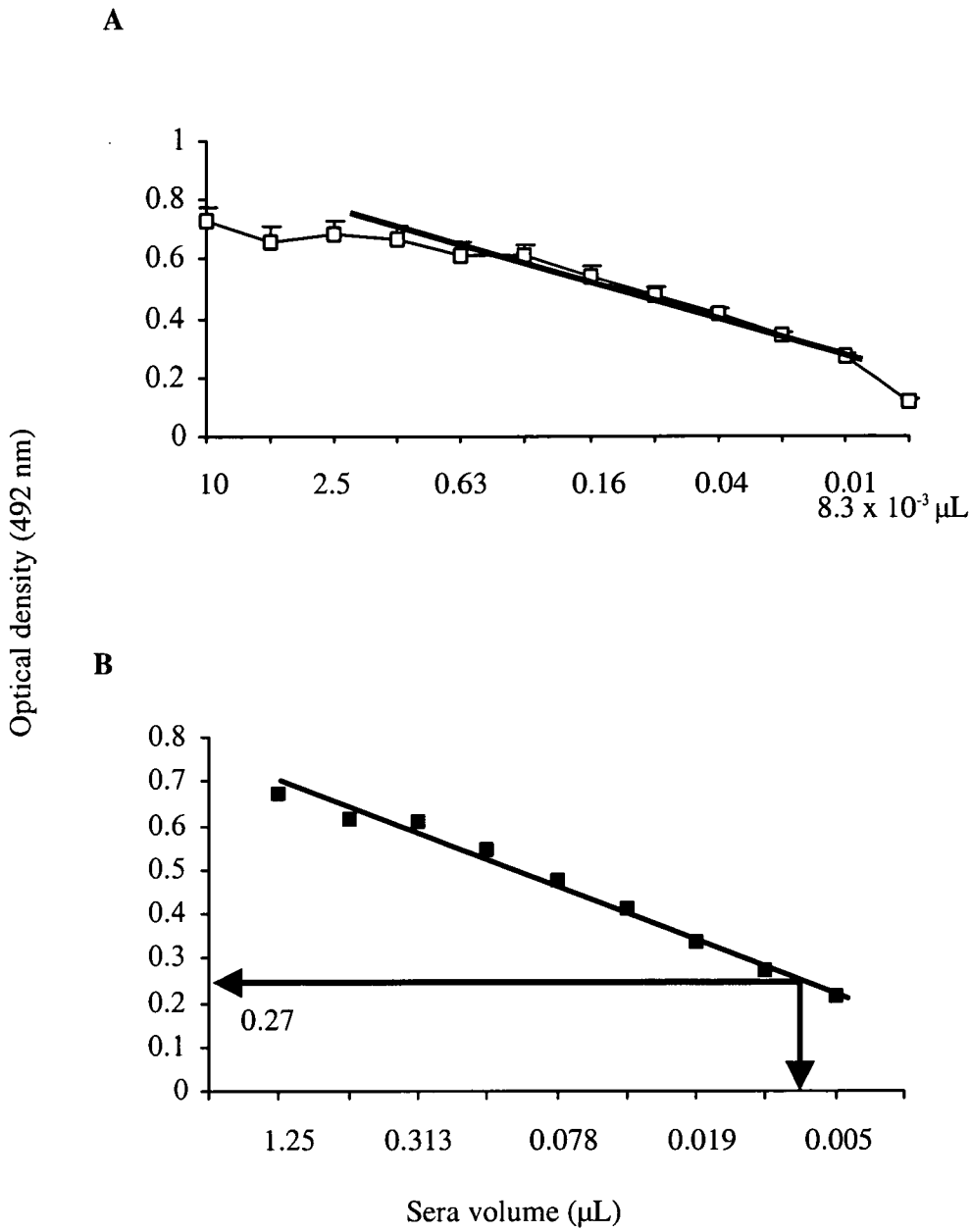

Figure 5.3 Titration curve and linear regression of barramundi anti-*V. harveyi* (strain no. 9050405) pooled antisera. **A.** Antisera titrated through dilution series 1:5 to 1:10240 against *V. harveyi* sonicate (strain no. 9050405) coated at 5.5µg mL^{-1} (protein). **B.** Regression of linear portion of curve ($r^2 = 0.983$) showing the volume of sera giving 40% of the maximum optical density from which a unit of antibody activity is derived.

5.2.5 Statistical analyses

All data were analysed with a one-way analysis of variance (ANOVA) using the JMP 3.2.1 statistical package. ANOVA assumptions of data normality and homogeneity of variances were tested using the Shapiro-Wilk W test and Bartlett's test respectively. Nested analyses were used to ascertain differences in replicate aquaria within treatments and due to time (i.e. sampling times of 10 and 21 days post booster), thus factors of aquaria and time were nested within treatment for analyses. Significant differences between treatment groups were highlighted using multiple comparisons of means (Tukey-Kramer HSD test). Phagocytic index data were not normally distributed which a common logarithmic transformation failed to rectify ($P = 0.046$), therefore a non-parametric test (Kruskal-Wallis test) was applied. Similarly, serum *V. harveyi* inhibitory activity data, due to a large number of zero values, fulfilled neither assumptions of normality nor homogeneity of variances, thus the same non-parametric test was used. Antibody activities between treatment groups and after primary and secondary immunisations were compared using a t test for paired comparisons. In examining associations between specific antibody activity and *V. harveyi* inhibitory activity correlation coefficients were determined and tested for significance (Sokal and Rohlf, 1987). When examining differences in all parameters between aquaria or treatment groups at 10 and 21 days post booster, data were pooled if analyses had P values > 0.1. For all tests a significance level of $P < 0.05$ was adopted .

5.3 RESULTS

Specific humoral responses were noted in all bacterin-immunised groups after both primary and secondary immunisations (Figures 5.4 & 5.5). In both instances some anti-*V. harveyi* activity was noted in control (saline-immunised) fish, however this activity was only found in five individual fish after both primary and secondary immunisations (Figures 5.4A and 5.5A). There was no difference in antibody activity between tanks within treatment groups ($P = 0.35$) or at 10 and 21 days after secondary immunisations ($P = 0.19$), thus data were

pooled. Analyses of antibody activity between groups 21 days after primary immunisation showed that only the IP bacterin-immunised group was significantly higher than its saline-treated control. However, there was no significant difference between IMM and AI bacterin-immunised groups and their controls, although there was a trend for higher antibody activity in the bacterin-treated groups. A similar pattern occurred 21 days after secondary immunisation however, for the bacterin-immunised fish antibody activity was significantly higher in the IP group compared to IMM and AI groups ($P < 0.001$). Antigen specific antibody activity was more consistent in the bacterin treated groups with the majority of fish responding whereas control groups had a small percentage of responders (Table 5.2).

Table 5.2 Number of individual barramundi displaying anti-*V. harveyi* antibody activity, as determined by ELISA, in serum after primary and secondary immunisations with *V. harveyi* bacterin or saline via intraperitoneal injection (IP), immersion (IMM) or anal intubation (AI)

Treatment	Administration method	Number of individual fish displaying anti-*V. harveyi* antibody activity:	
		1° immunisation	2° immunisation
Bacterin	IP	18/20 (90%)	20/20 (100%)
	IMM	5/18 (27%)	8/20 (40%)
	AI	7/20 (35%	8/20 (40%
Saline	IP	1/19 (5%)	1/20 (5%)
	IMM	0/20 (0)	1/20 (5%)
	AI	4/20 (20%)	1/20 (5%)
Naive		n.a.	2/20 (10%)

n.a. not applicable as naïve group were only sampled at the end of the experiment

The increase in antibody activity observed in the IP group after the secondary immunisation (Figure 5.6) was significantly higher than activity noted after the primary immunisation ($P < 0.001$), which may be indicative of memory induction. Similar comparisons in the IMM ($P = 0.22$) and AI ($P = 0.48$) groups showed no significant increase in antibody activity after secondary immunisations.

Bacteriostatic activity of barramundi serum, expressed as percentage inhibition of *V. harveyi* relative to pooled non-immune standard barramundi sera, was observed in all bacterin-immunised groups as well as in sera of some individuals in control groups (Figure 5.7A & B). This activity was however, significantly higher and less variable in the IP bacterin group ($P < 0.001$) with all individuals displaying substantial inhibition of *V. harveyi* and 16 of 18 fish recording greater than 60%. Inhibitory activity was generally much lower and more varied in other groups with 50% or less (Table 5.3) of individuals showing *V. harveyi* inhibitory activity as assessed by this assay. There was no significant difference within groups at 10 and 21 days post booster ($P = 0.25$), therefore data were pooled.

Examination of the relationship between serum anti-*V. harveyi* antibody activity and inhibitory activity found no correlation (Figures 5.8 and 5.9) However, sera from IP-immunised fish was consistently high in bacterial inhibitory activity and had variable specific antibody activity whereas sera from immersed and anally intubated displayed activity for both parameters in only some of the fish sampled. Analyses of all mucus samples taken from the hind gut and the skin failed to show either specific antibody activity or *V. harveyi* inhibitory activity.

There was no real trend evident in the non-specific parameters examined with no difference in serum lysozyme activity between any treatment groups ($P = 0.93$) (Figure 5.10A). Although phagocytic indices of anterior kidney macrophage suspensions showed some statistically significant differences (Figure 5.10B), there was no enhancement of this parameter between treatment groups.

Table 5.3 Number of individual barramundi displaying *V. harveyi* inhibitory activity in serum after immunisation with *V. harveyi* bacterin or saline via intraperitoneal injection (IP), immersion (IMM) or anal intubation (AI)

Treatment	Administration method	Number of individual fish displaying *V. harveyi* inhibitory activity
Bacterin	IP	18/18 (100%)
	IMM	9/18 (50%)
	AI	6/15 (40%)
Saline	IP	5/14 (35%)
	IMM	6/14 (42%)
	AI	7/18 (38%)
Naive		3/9 (30%)

No enhancement of non-specific immune parameters were noted in this experiment. Phagocytic indices (Figure 5.10A) of head kidney macrophage suspensions indicated no macrophage activation between fish immunised via various methods with either bacterin or saline. In some cases, phagocytic indices were significantly lower ($P < 0.001$) 21 days post secondary immunisations in AI (both bacterin and saline) and naive fish than in IP and IMM fish. Similarly serum lysozyme activity (Figure 5.10B) showed no significant enhancement ($P = 0.13$) between or within treatment groups at 10 and 21 after secondary immunisations ($P = 0.12$).

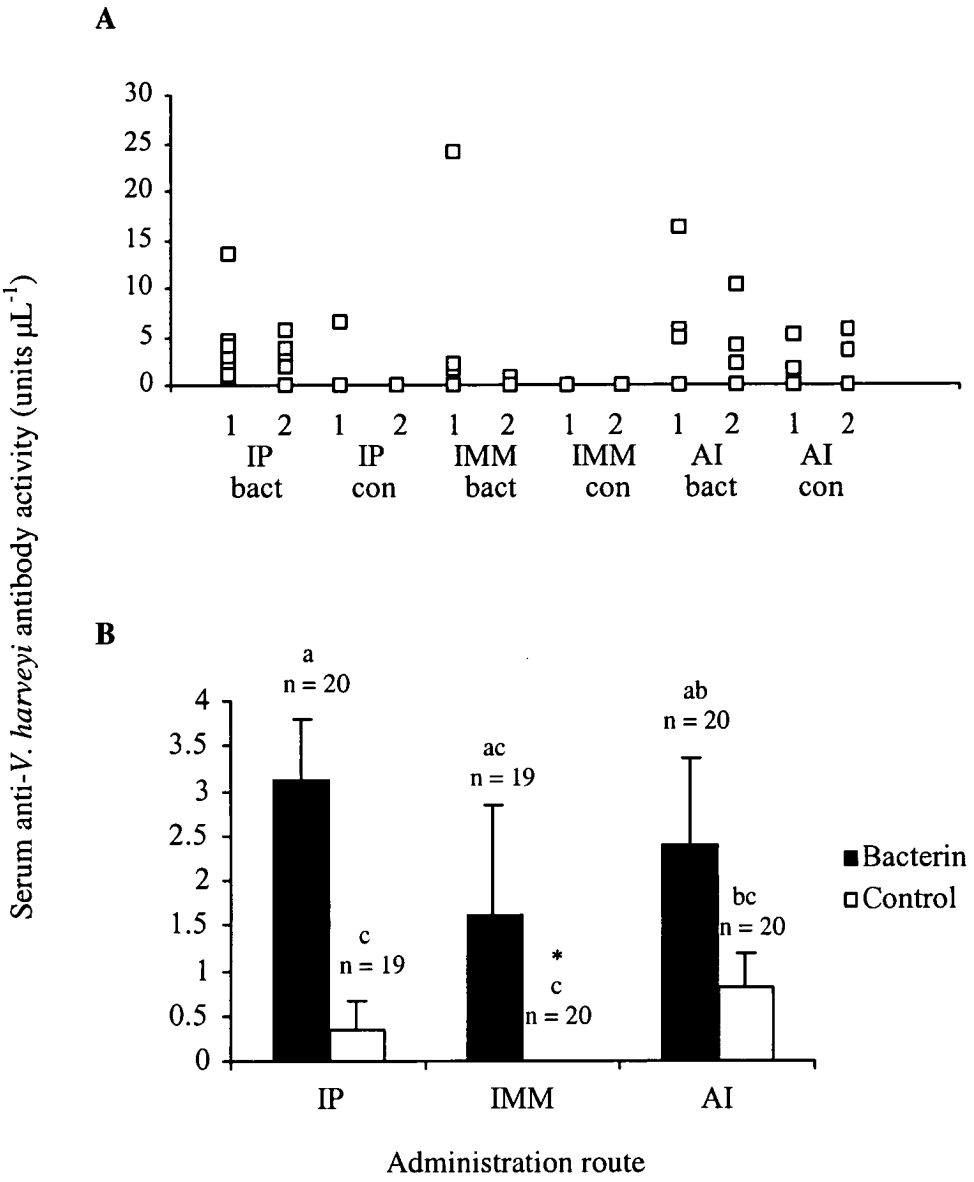

Figure 5.4 Specific anti-*V. harveyi* antibody activity of barramundi serum 21 days after primary immunisation with *V. harveyi* bacterin or saline via intraperitoneal injection (IP), immersion (IMM) or anal intubation (AI). **A.** Individual fish within 2 aquaria per treatment group of bacterin (bact) or saline control (con). **B.** Fish per treatment group, values are means + SE and disparate superscripts show significant differences (P = 0.016, Tukey-Kramer HSD test) * IMM control group displayed no activity.

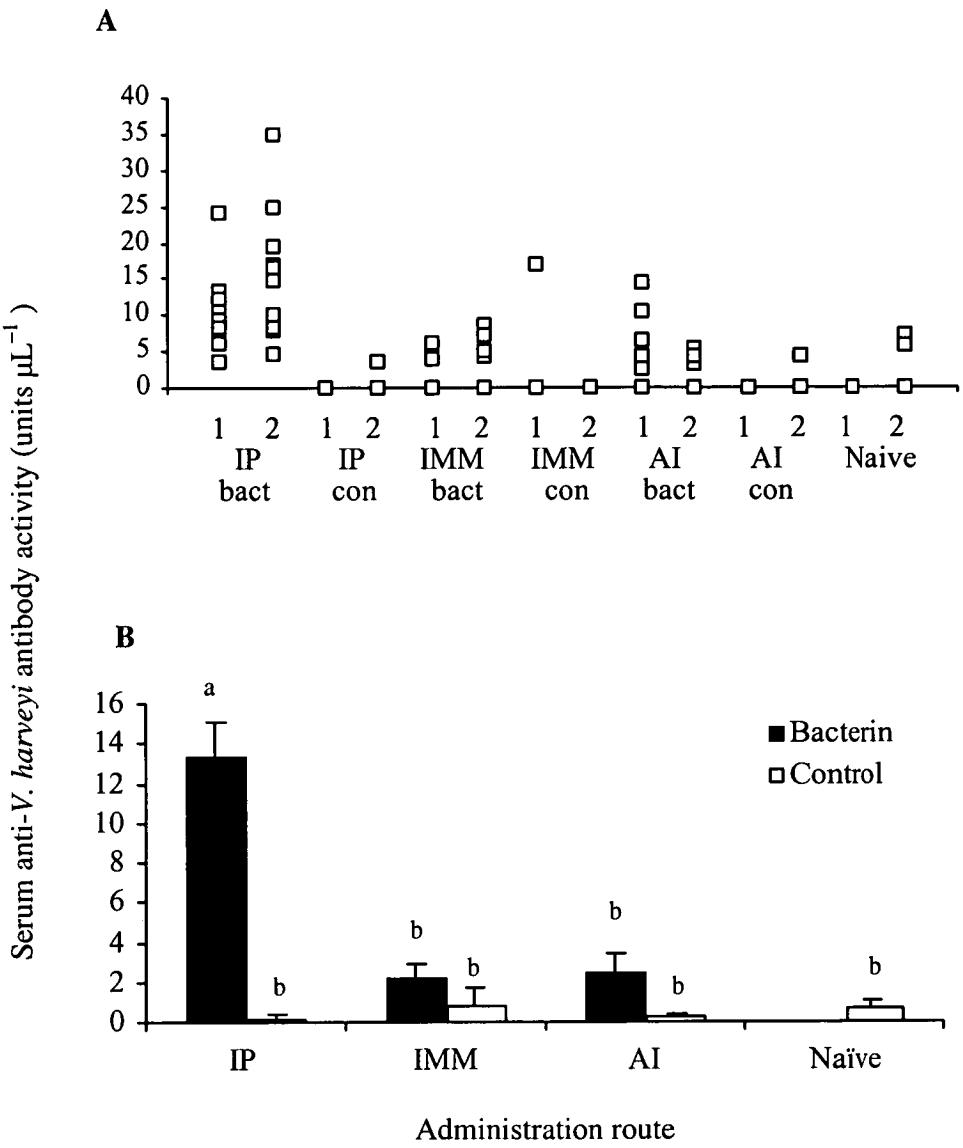

Figure 5.5 Specific anti-*V. harveyi* antibody activity of barramundi serum after booster immunisation with *V. harveyi* bacterin or saline via intraperitoneal injection (IP), immersion (IMM), anal intubation (AI) or naive to immunisation and handling. As no difference was detected between sampling periods of 10 and 21 days data were pooled. **A.** Individual fish within 2 aquaria per treatment group of bacterin (bact) or saline control (con). **B.** Fish per treatment group. Values are means + SE and disparate superscripts show significant differences ($P < 0.001$, Tukey-Kramer HSD test, $n = 20$).

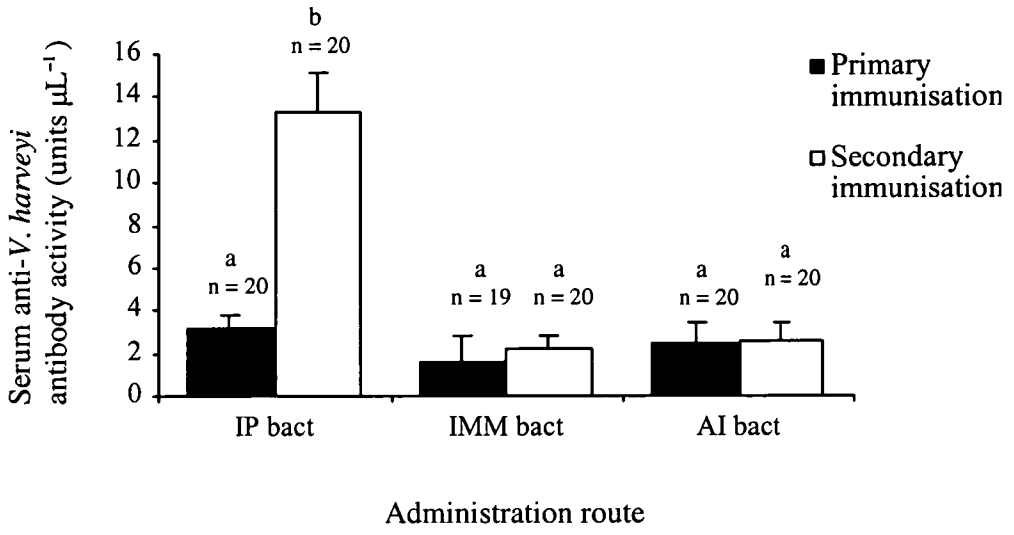

Figure 5.6 Specific anti-*V. harveyi* antibody activity of barramundi serum immunised with *V. harveyi* bacterin (bact) via intraperitoneal injection (IP), immersion (IMM) and anal intubation (AI) after primary and secondary immunisations. Values are means + SE, and disparate superscripts show significant differences ($P < 0.001$, paired t Test).

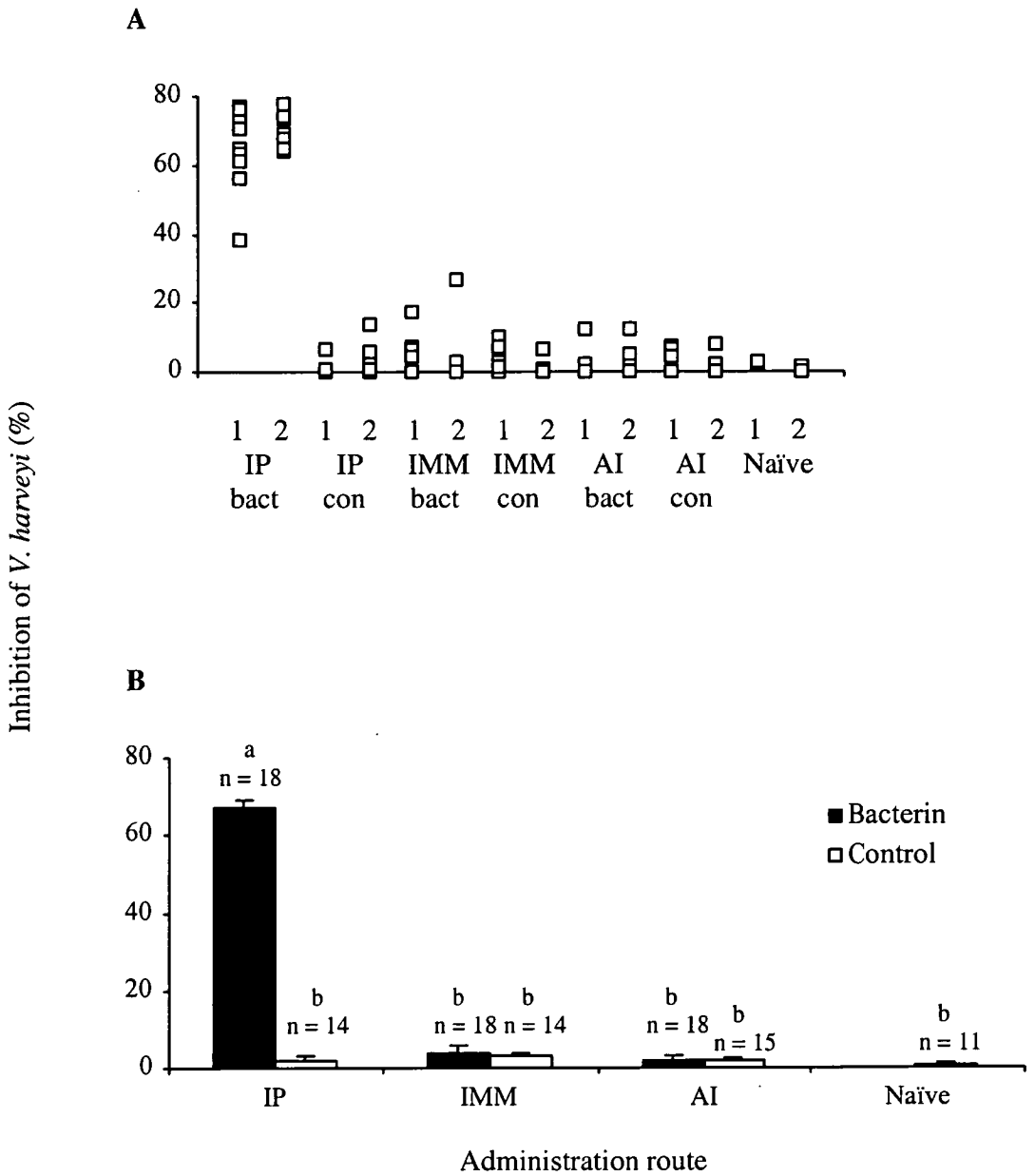

Figure 5.7 *V. harveyi* inhibitory activity of barramundi serum (relative to pooled non-immune standard sera) after immunisation with *V. harveyi* bacterin via intraperitoneal injection (IP), immersion (IMM) and anal intubation (AI) 21 days after secondary immunisations. Data were pooled as there was no difference between 10 and 21 days post booster **A.** Individual fish within 2 aquaria per treatment group of bacterin (bact) or saline control (con). **B.** Fish per treatment group, values are means + SE and disparate superscripts show significant differences ($P < 0.001$, Kruskal-Wallis test).

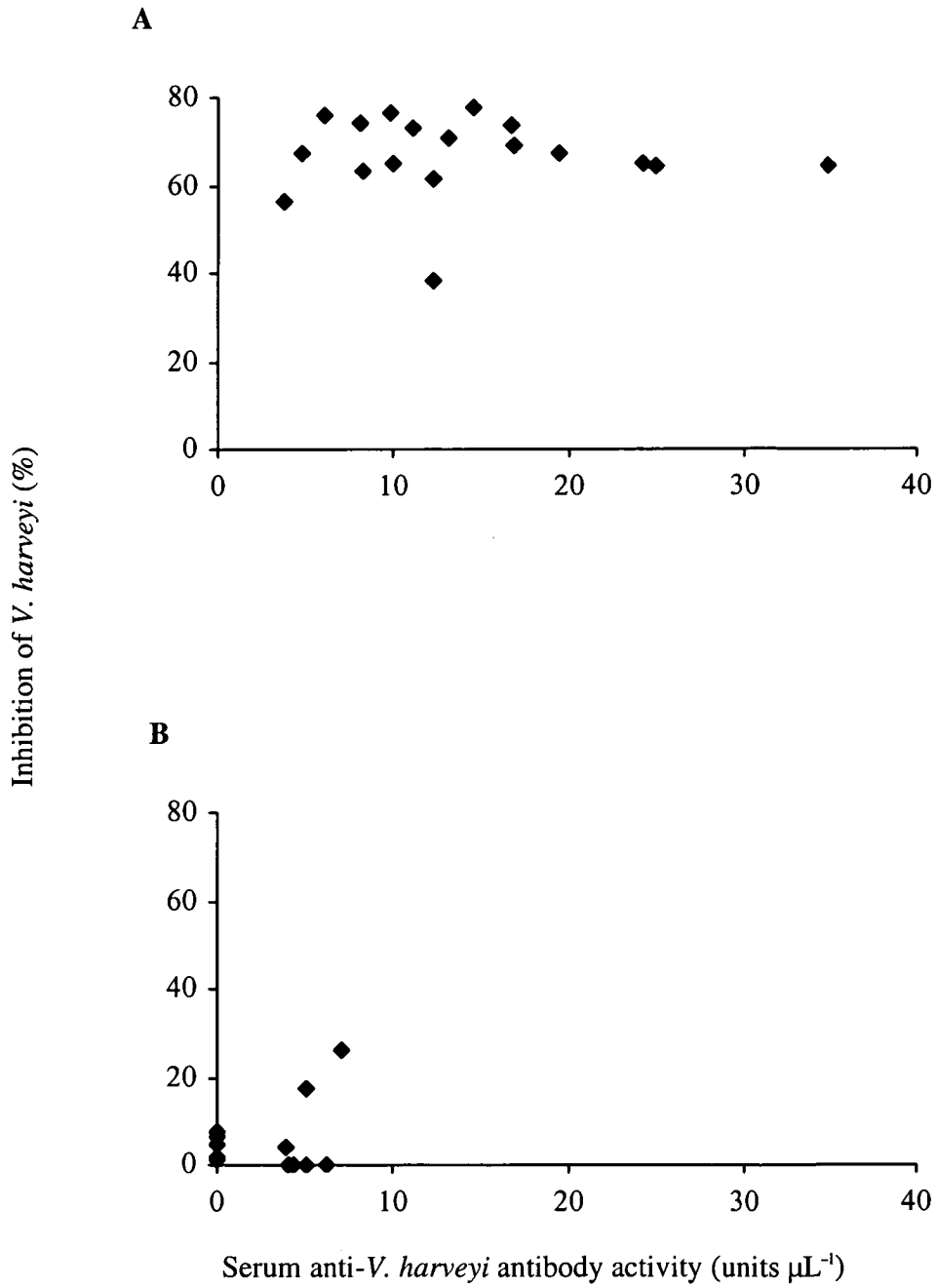

Figure 5.8 Relationship between barramundi serum anti-*V. harveyi* antibody activity and serum *V. harveyi* inhibitory activity (relative to pooled non-immune standard sera) showing no significant correlation ($P > 0.05$) when examined by individual treatment group. **A.** Bacterin administered by intraperitoneal injection ($r = 0.041$, $n = 18$). **B.** Bacterin administered by immersion ($r = 0.334$, $n = 12$).

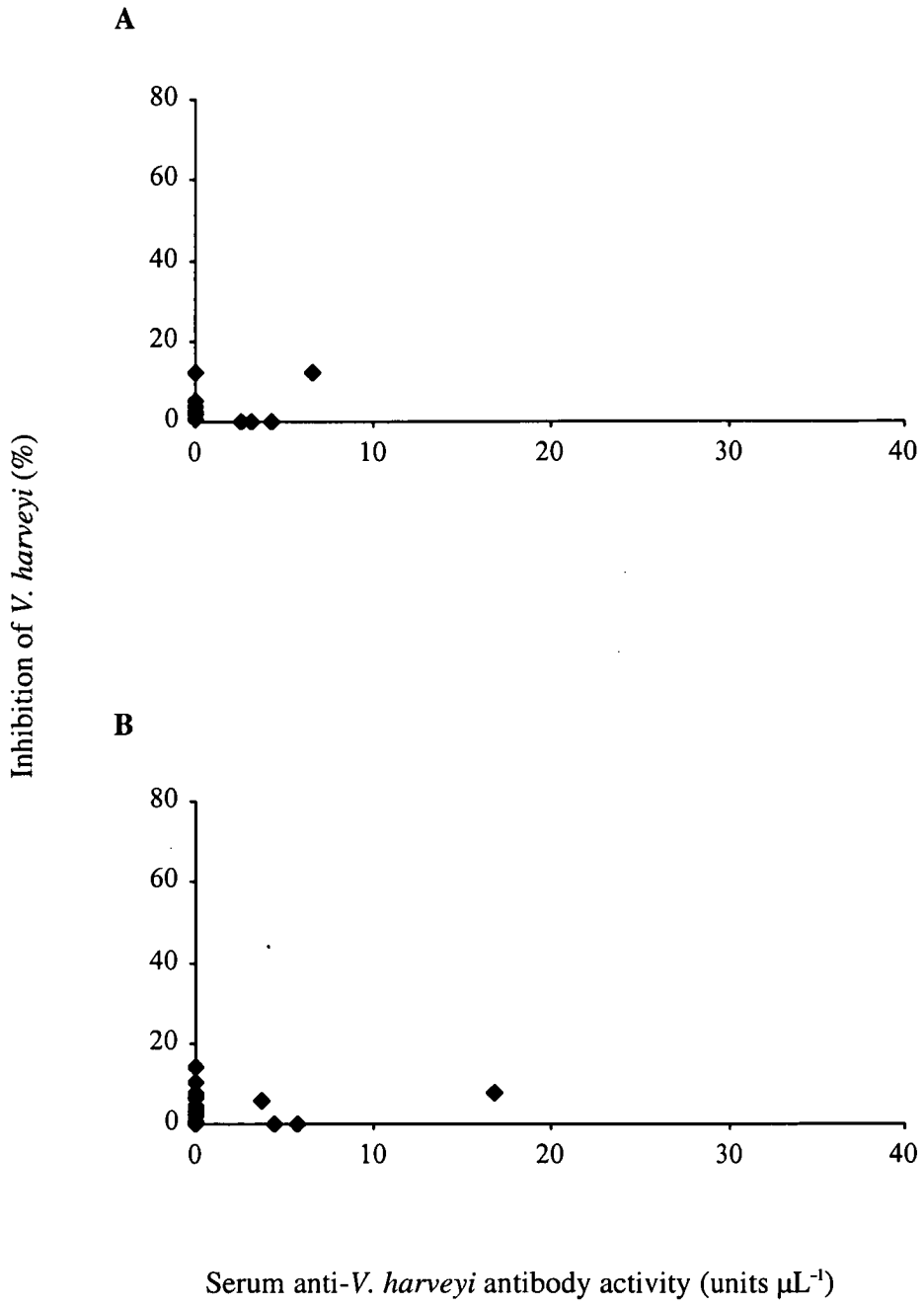

Figure 5.9 Relationship between barramundi serum anti- *V. harveyi* antibody activity and *V. harveyi* inhibitory activity (relative to pooled non-immune standard sera) showing no significant correlation ($P > 0.05$) when examined by individual treatment group. **A.** Bacterin administered by anal intubation ($r = 0.161$, $n = 10$). **B.** Individuals from saline treated control groups displaying bacteriostatic and/or anti- *V. harveyi* antibody activity ($r = 0.077$, $n = 23$).

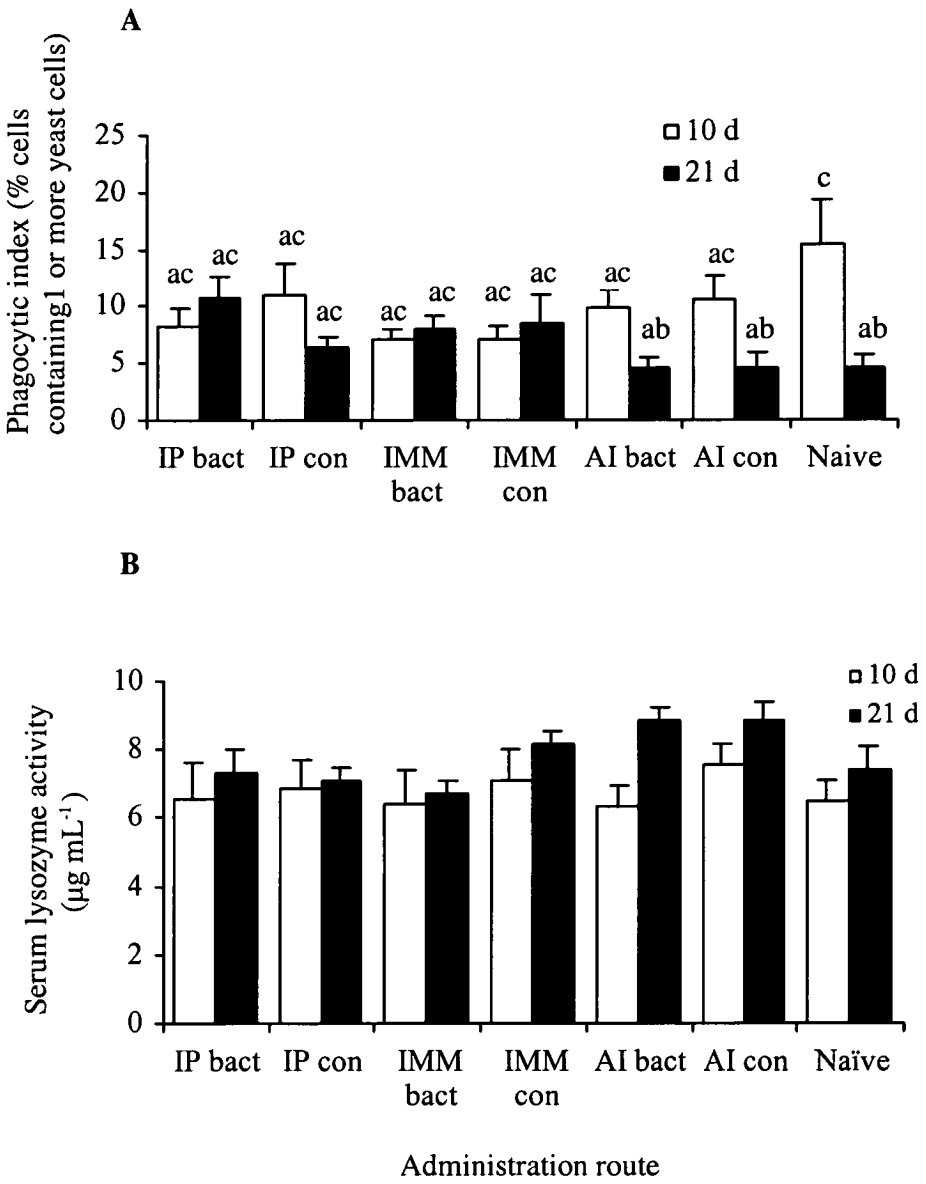

Figure 5.10 Non-specific immune parameters in barramundi 10 and 21 days after secondary immunisations with *V. harveyi* bacterin (bact) or saline control (con) via intraperitoneal injection (IP), immersion (IMM), anal intubation (AI) or naive to immunisation and handling. **A.** Phagocytic indices of anterior kidney macrophage suspensions, all values are means + SE (n = 10) and disparate superscripts show significant differences ($P < 0.001$, Kruskal-Wallis test) **B.** Serum lysozyme activity, all values are means + SE (n = 10) (no significant difference, $P = 0.93$).

5.4 DISCUSSION

This study has shown that barramundi respond systemically, in terms of antibody, to whole-killed bacterial cell antigens from *V. harveyi* when administered by IP injection, immersion or anal intubation, although a similar mucosal response was not detected. It also provides more evidence possibly supporting the existence of immunological memory, given the four-fold increase in specific antibody activity after secondary IP administration of the bacterin. Furthermore, a correlation between specific antibody activity and bacterial inhibitory activity was noted when fish across all treatment groups are examined, although not when individual groups were analysed.

Induction of systemic antibody after peranal bacterin delivery demonstrates the success of antigen uptake in the posterior intestine of barramundi, and therefore indicates that the potential of orally delivered vaccines is worth investigating. Previous studies have shown systemic antibody responses after peranal (Rombout *et al.*, 1986; Joosten *et al.*, 1986; Palm *et al.*, 1998; Cain *et al.*, 2000) and peroral (Ainsworth *et al.*, 1995; Palm *et al.*, 1998) antigen delivery, although reports of protection from the gut delivery route show that it provides less protection in comparison to that provided after immersion or IP injection delivery. Oral delivery of *V. anguillarum* bacterin to ayu, *Plecoglossus altivelis*, has been protective against vibriosis (Kawano *et al.*, 1984; Kawai and Kusuda, 1995) but protection was limited by time.

The apparent anti-*V. harveyi* antibody activity noted in some individuals in control groups may be due to previous exposure of controls to the bacterium, cross-reactivity of existing antibodies to *V. harveyi* antigens or, inaccuracies and lack of specificity of the ELISA. Inclusion of pooled non-immune standard sera on every plate and the consistent lack of antibody activity in this standard probably rules out the latter explanation. Previous exposure to *V. harveyi* is possible given the global distribution of the bacterium. An equally likely explanation is cross-reactivity of existing antibodies with *V. harveyi* antigens. Immunoblots have shown reactivity of serum antibody from non-immunised barramundi with

lipopolysaccharide (LPS) from *Escherichia coli* and with sonicated cells of *V. harveyi* (Bridle, 2000). This suggests that there may be common epitopes within the LPS of Gram negative bacteria to which some barramundi displayed specific antibody. If this is the case then some bacterin-treated fish may also have had cross-reactive antibodies which would be indistiguishable by the ELISA. Regardless of whether the specific antibody in control fish was due to previous exposure to *V. harveyi* or cross-reactive antibodies, the impact of such a scenario complicates the interpretation of the results. However, even though for IMM and AI treatment groups there were no statistically significant differences in mean antibody activity in bacterin and saline treatments, there was a trend for higher activity in the bacterin-treated fish where a higher proportion of individuals responded. The fact that the majority of individual fish responded in terms of specific antibody to the bacterin whereas only a small proportion of saline-immunised fish displayed antibody activity would suggest that the antibody response to the bacterin was specific.

The administration route-dependent increase in serum antibodies observed in this study concurs with findings of Jenkins *et al.* (1994) who reported higher levels of plasma antibody after IP delivery of human gamma globulin to tilapia, *Oreochromis mossambicus*, than after either anal or oral delivery. More recently, Palm *et al.* (1998) reported little difference in serum antibody titers of rainbow trout, *O. mykiss*, after IP and IMM delivery of *V. anguillarum* bacterin and these titers were substantially higher than those recorded in the majority of fish after oral bacterin delivery. Additionally, these investigators found little difference in survival after bacterial challenge between all immunised groups. In this case it seems that low levels of specific antibody are sufficient for protection and it is possible that there is a cut off point where higher antibody levels do not lead to increased protection. These are interesting results as they seem to contradict observations on antibody responses made in the present study and previously expressed views that the degree of protection depended on the bacterin administration route or procedure (Midtlyng *et al.*, 1996a; Thune *et al.*, 1997; Nakanishi and Ototake, 1997; Quentel and Vigneulle, 1997). Differences in observations may be due to challenge methods, although challenges in these studies were water–borne, some were via cohabitation (Midtlyng *et al.*, 1996b) and others by adding virulent cells to the water (Thune *et al.*, 1997).

A particularly pertinent finding of this study was that all the IP bacterin-treated fish displayed a serum antibody response and these sera displayed substantial *V. harveyi* inhibitory activity regardless of the magnitude of specific antibody activity. Firstly, this may indicate that the bacterin elicited a potentially protective response, at least in the specific humoral arm of the immune system. Secondly, this response may not be a function of the magnitude of the antibody response, as suggested by the lack of correlation between the antibody activity and the bacterial inhibitory activity. As alluded to above, it may be that antibody is responsible for the bacterial inhibitory activity but a saturation effect may occur where more antibody activity does not increase inhibitory activity. Given the data and the means by which it was collected it is not possible to discern a causal link between antibody activity and bacterial inhibitory activity.

Although other humoral serum factors such as complement, lysozyme and C-reactive protein have antibacterial properties (Yano, 1996), these are thought not to be responsible for the bacterial inhibitory activity observed in this experiment. Complement has known bactericidal activity for a range of teleosts and the classical complement pathway is activated by the antibody-antigen complex (Sakai, 1992). However, the bacteriostatic assays were conducted with heat-treated, and consequently complement-inactivated, serum samples as well as untreated samples with no difference noted. It could be argued that complement would be inactivated in the untreated samples as well given components that are heat labile over time and even at temperatures of -20°C (Sakai, 1992). It is therefore unlikely that complement was involved in *V. harveyi* inhibitory activity. There is also solid evidence for the lack of involvement of lysozyme in the observed inhibitory activity. Firstly, in the absence of a complement-derived membrane attack complex to initially disrupt the bacterial cell walls, lysozyme would be less effective against Gram negative bacteria (Yano, 1996), though in rainbow trout a lysozyme variant, designated type 11, was found to act directly on four Gram negative bacterial fish pathogens at physiological serum concentrations (Grinde, 1989). Secondly, there were no differences detected in serum lysozyme activity between treatment groups within this study. Other serum factors such as C-reactive protein (CRP) and other acute phase proteins would probably only be in circulation at basal levels given

the time between immunisation (primary and secondary) and sampling, unless there was some external injury or inflammation. CRP does activate humoral defences via complement activation (Ellis, 1999b) and when isolated from rainbow trout has been shown to inhibit *in vitro* growth of *V. anguillarum* (Nakanishi *et al.*, 1991).

Although there was no correlation between antibody activity and bacterial inhibitory activity it is interesting that IP-immunised fish displayed high levels of inhibitory activity and varying levels of antibody activity. It may be that relatively low levels of anti-*V. harveyi* antibodies are sufficient to inhibit *V. harveyi* in the *in vitro* assay system used. It has been shown previously that protection does not always correlate with antibody activity, indeed, protection against bacterial challenge has been reported in immunised fish demonstrating no systemic antibody activity (Ellis, 1988a). It may be that small quantities of antibody are sufficient or that cell-mediated mechanisms are partially responsible. In this experiment, activity noted in the serum alone rules out direct involvement of the cell-mediated arm.

A clear way to elucidate the role of antibody in bacterial inhibitory activity and protection would be by passive immunisation then challenge with live bacteria. Unfortunately it was not possible to challenge barramundi with virulent *V. harveyi* in our laboratory, due to quarantine regulations.

In this experiment the bacterin intubated into the hind gut of the fish consisted of washed formalin-killed whole cells, perhaps one reason for the lower response compared to the IP treated fish was the lack of sufficient quantity of soluble factors in the bacterin. A previous experiment using *V. anguillarum* found that phagocytosis and uptake of whole cells by enterocytes did not occur in the hind gut of rainbow trout and carp whereas antigen uptake was observed in vaccine culture supernatant and purified lipopolysaccharide (Joosten *et al.*, 1996.) On the other hand, Agius *et al* (1983) found better protection after challenge in rainbow trout immunised perorally with whole-killed *V. anguillarum* cells than with an extract of centrifuged culture, whether whole cells, particles or soluble antigenic determinants were taken up in the appropriate section of the gut is not clear. Similarly, Palm *et al.* (1998) found that oral delivery of whole-killed *V. anguillarum* cells conferred

protection in a laboratory challenge, again the nature of the antigen taken up was not investigated.

In terms of antibody production antigen dosage to the hind gut appears important in determining the magnitude of the response. The present study found only a minimal antibody response after an initial and booster anal immunisation with bacterin. Whereas Rombout *et al.* (1989) found repeated doses of ferritin delivered anally or orally, in association with food, elicited anti-ferritin antibody levels in carp plasma comparable to those reached after intramuscular injection. Jenkins *et al.* (1994) reported a considerable specific antibody response in the plasma of tilapia, *O. mossambicus,* after a single oral or anal immunisation with human gamma globulin (HGG). However, the antigen was delivered with the saponin adjuvant Quil-A (Jenkins *et al.* ,1994).

None of the non-specific immune responses quantified showed any difference between any of the treatment groups. Serum lysozyme activity indicated that there was no enhancement or, that it was resolved by the time of sampling. Similary, head kidney macrophage phagocytic ability showed generally no enhancement, however phagocytic activity in the naïve group was significantly higher at 10 days post booster immunization than at 21 days in groups anally intubated with bacterin or saline and the naïve group. A possible explanation is that the phagocytic activity may have been resolved by the time 21 days had elapsed. Macrophages, though easily stimulated *in vitro*, may have passed the activation stage.

The results presented here show that barramundi respond systemically to *V. harveyi* bacterin when it is delivered via IP injection, exposure to GALT and immersion and that in accordance with many authors the response was highest in injected fish relative to intubated and immersed fish.

CHAPTER 6

THE ROLE OF LIPOPOLYSACCHARIDE FROM *Vibrio harveyi* AS AN IMMUNOGEN AND PROTECTIVE ANTIGEN COMPARED TO BACTERIN

6.1 INTRODUCTION

Lipopolysaccharide (LPS) is a constituent of the outer cell membrane lipid bilayer of Gram negative bacteria which has both toxic properties and immunomodulating activities. It consists of three important regions, the outer oligosaccharide side chain responsible for O-antigenic specificity of individual species, a core polysaccharide common to virtually all Gram negative bacteria and, an inner lipid A structure (Burrell, 1990). A smaller form of LPS which lacks the O-antigen is designated rough (R) type whereas LPS with the O-antigen is designated smooth (S) type (Sprott *et al.*, 1994). Many strains of bacteria produce both LPS types, in varying proportions, but some strains only produce the R-type (Sprott *et al.*, 1994). LPS is also known as endotoxin which is the naturally occurring LPS macromolecule with associated membrane or cytoplasmic proteins (Burrell, 1990). The lipid A structure is thought to be primarily responsible for endotoxic activity of the molecule, although other structures have biologically stimulating functions (Burrell, 1990). Immunomodulatory effects of LPS are well documented and the molecule interacts with numerous humoral and cellular factors (Morrison and Ryan, 1979), including alternate complement pathway activation by oligosaccharide side chain (Morrison and Kline, 1977), influencing levels of C-reactive protein and cortisol in plaice, *Pleuronectes platessa* L., (White *et al.*, 1984) and stimulation of mammalian macrophages to produce and release immunoregulatory factors such as prostaglandin (Kunkel *et al.*, 1986) and tumor necrosis factor (TNF-α) (Sayers *et al.*, 1987). In mammals, LPS is a powerful B lymphocyte mitogen as well as being an activator which are seemingly separable roles (Burrell, 1990). This independence was demonstrated by Haeffner-Cavaillon *et al.* (1982) when they reported that

polysaccharide rich fractions were not mitogenic to rabbit splenocytes but did induce Ig synthesis in the B cell population.

Although LPS can be very toxic in some mammals causing severe hypotension and death, fish are reportedly less susceptible to these toxic effects (Berczi *et al.*, 1966; Wedemeyer and Ross, 1969; Paterson and Fryer, 1974; Harbell *et al.*, 1979). In fact the immunomodulating activities of LPS can have a beneficial effect on the immune response by up-regulating non-specific immune parameters such as respiratory burst activity of mammalian macrophages (Pabst and Johnston, 1980). In fish, Solem *et al.* (1995) demonstrated the ability of LPS from *Aeromonas salmonicida* to stimulate *in vitro* macrophage respiratory burst and phagocytic activity in Atlantic salmon, *S. salar*, Similarly, enhancement of phagocytic activity of leucocytes in the eel, *Anguilla japonica*, after injection of LPS from *Edwardsiella tarda* has been reported (Salati *et al.*, 1987). More specifically, antibodies and agglutinins have been induced in brown trout, *Salmo trutta*, after immunisation with LPS from *Salmonella typhimurium* (Ingram and Alexander, 1980). Nakhla *et al.* (1997) found that rainbow trout, *Oncorhynchus mykiss*, generated anti-LPS activity in serum after repeated injections with LPS from *A. salmonicida* and that this activity was prolonged when LPS was incorporated into liposomes.

On a more holistic level, immunisation of fish with crude preparations of LPS has been shown to be protective against some bacterial diseases (Baba *et al.*, 1988; Rogers, 1990). Thus LPS is very immunogenic in fish and is the main surface antigen of the pathogenic vibrios (Chart and Trust, 1984) Lipopolysaccharide is thought to be the protective antigen of the fish pathogenic bacteria *V. ordalii* (Velji *et al.*, 1990; Velji *et al.*, 1991), *V. anguillarum, V. salmonicida* and *Y. ruckeri* (Press and Lillehaug, 1995). Similarly, Al-Harbi and Austin (1992a) found injected LPS extracted from a *Cytophaga*-like bacterium to be protective in turbot, *Scopthalamus maximus*, against challenge from the bacterium.

In the context of a vaccine component, it is recognised in mammals that immunisation with a bacterial vaccine or isolated endotoxin results in antibody directed primarily against the O-antigen (the repeating oligosaccharide) component of LPS (Morrison and Ryan, 1979).

However, LPS is considered to be a thymus independent (or T-helper cell independent) antigen and induces antibody responses without the activation of T cells and with little immunological memory induction (Roitt *et al.*, 1996). Lipopolysaccharide from an *A. salmonicida* vaccine has been shown to be present in spleen and head kidney melanomacrophages 16 weeks after intraperitoneal injection of vaccine (Press *et al.*, 1996). These authors suggest that such retention of antigen in lymphoid organs such as the spleen and head kidney may be important in the formation of immunological memory. Though retention of antigen itself does not necessarily imply a protective immune response in fish (Press *et al.*, 1995) the persistence of the antigen within the fish does play a role, along with dose and administration route, in the magnitude of immunological memory (Rijkers *et al.*, 1980). These processes in fish are not yet fully understood but it seems possible that LPS may be retained within lymphoid tissue macrophages and thus play a role in prolonged protection and consequently be an antigen worth considering as a vaccine component.

Along with a fuller understanding of the processes involved in the vaccine-induced disease protection there is a need to identify the protective components of vaccines so that they may best be administered to the fish. To elucidate what effect *V. harveyi* LPS may have on barramundi this study compares the effect of immunisation with bacterin and LPS in terms of some non-specific responses and antibody responses.

6.2 MATERIALS AND METHODS

6.2.1 Fish husbandry

Procurement and husbandry of barramundi were as described in section 3.2, although the acclimation period was 4 weeks and there was one aquarium per treatment, each containing 10 fish. Before immunisations aquaria were randomly allocated a treatment. A selection of fish naive to all treatments, including placebos, served to provide a pool of non-immune sera

in lieu of pre-bleeds. The fish were weighed at the commencement of the trial and the mean weight of each treatment group appears in Table 6.1

Table 6.1 Mean weight (± SE; n = 12) of barramundi in each treatment group at the commencement of the immunisation experiment using *V. harveyi* bacterin, lipopolysaccharide (LPS) or saline delivered by intraperitoneal injection (IP)

Treatment group	Mean weight (g)
Bacterin	40.3 ± 2.2
LPS	52.7 ± 3.5
Saline	42.9 ± 7.7

6.2.2 Bacterin Preparation and administration

The bacterin was prepared as described in section 3.3 and, as was the case in chapter 5, *V. harveyi* strain no. 9050405 was used as the culture bacterium. Anaesthetised fish were injected intraperitoneally with 0.13 mL inocula for a dose of 0.08 mg (wet weight bacterial cells) per gram of fish.

6.2.3 Lipopolysaccharide extraction and administration

For isolation of LPS *V. harveyi* was grown in NB2 as 1 L volumes in 3 L flasks in a shaking water incubator at 30°C for 6.5 h. Lipopolysaccharide was then extracted from bacterial cells by a procedure modified by Sprott *et al.* (1994) from that of Galanos *et al.* (1969). This procedure required the harvesting of *V. harveyi* in exponential growth phase, so that the majority of cells would be robust and not damaged. To ensure cultures were in exponential growth phase after an incubation period of 6.5 h, cell densities were monitored every 0.5 h by measuring optical density at 550 nm on a spectrophotometer (UV-1200 series, Shimadzu). Briefly, the extraction procedure was as follows: 6 x 1 L cultures were chilled to

4°C and cells harvested by centrifugation at 100 x *g* for 10 min. Pellets were resuspended with a homogeniser in 50 mL of cold deionised water, then again centrifuged for 10 min at 12000 x *g*. Cells were then suspended in 1 mL of cold deionised water before being homogenised in 50 mL of cold methanol and centrifuged again for 10 min at 8000 x *g*. The pellet was then suspended in 50 mL of cold acetone, homogenised and centrifuged as above. The acetone washing step was repeated and the pellet was lyophilised overnight. LPS was extracted by combining 25 mL of a phenol (13.3% v v^{-1}), chloroform (33.3% v v^{-1}) and petroleum ether (53.3% v v^{-1}) mixture (PCP) with dried cells and homogenising for 30 s. Suspension was then centrifuged for 5 min at 9200 x *g*. Supernatant was collected and the pellet was resuspended in PCP and centrifuged as before. Supernatants were then combined and filtered through Whatman no. 1 paper then concentrated by evaporation under nitrogen gas at 50°C and centrifuged for 10 min at 12000 x *g*. The phenol liquid was aspirated with a syringe and needle and 10 mL of methanol added and suspension was centrifuged for 15 min at 12000 x *g*, methanol washing was repeated twice before the pellet was lyophilised overnight. The pellet was then suspended in 20 mL of 0.1 mM $MgCl_2$, homogenised and ultracentrifuged at 200000 x *g* for 4 h.

The total yield of LPS was approximately 1.5 mg. This was diluted in 3 mL of sterile saline (0.15 M NaCl) to give a final LPS concentration of 500 μg mL^{-1}. Aliquots from this stock were electrophoresed on SDS-PAGE and silver stained to check for purity and analysed for protein content with a Bradford microbioassay kit (Bio-Rad). The result was a protein concentration of 17 μg mL^{-1} or approximately 3%. Anaesthetised fish were immunised with 40 μg of LPS in sterile saline as 0.1 mL inocula. Control fish were injected with 0.13 mL of sterile saline.

Twenty one days later all fish were anaesthetised then re-immunised as above for bacterin but with 30 μg per fish of LPS, the mean fish weight was 71.2 ± 15.9 g.

For comparative purposes a crude preparation of LPS was extracted from *V. harveyi* by proteinase K digestion according to Hitchcock and Brown (1983). The bacterium was cultured to exponential growth as described above, then 1.5 mL of the suspension was

centrifuged in a microcentrifuge for 3 min. The pellet was resuspended in SDS-PAGE reducing sample buffer (section 2.2.2) and heated at 100°C for 10 min, after which 25 μg of proteinase K (Boehringer Mannheim) was added to the suspension which was then incubated at 60°C for 1 h. The preparation was then stored at –20°C until used.

6.2.4 Sampling procedures

Seven days after booster, half the fish (i.e. five individuals) from each group were lethally anaesthetised with benzocaine (200 mg L^{-1}) then serum and mucus (both skin and gut) samples taken prior to removal of head kidney and subsequent harvest of macrophages. Serum samples were allowed to clot overnight at 4°C then stored at –20°C. Mucus samples were collected and stored as previously described in section 3.4 as were head kidney tissues. Remaining fish were sampled as above at 21 days post booster.

6.2.5 Immune assays

Macrophages were harvested as described in section 3.5.1 then incubated with yeast cells to measure phagocytic activity (section 3.5.2). Assays were also performed to assess serum lysozyme activity, anti-*V. harveyi* antibody activity by ELISA and bacteriostatic ability (sections 3.5.3-3.5.5).

The ELISA was identical to that used for the experiment described in Chapter 5, and the same calculation was used to determine anti-*V. harveyi* antibody activity. Although ideally LPS should be the coating antigen to detect specific antibody in the LPS-immunised group of fish the poor yield meant there was insufficient LPS for use in ELISA calibration and subsequent processing of samples. It was therefore decided to use the same coating antigen in ELISA for all treatment groups.

6.2.6 SDS-PAGE and immunoblotting

Electrophoretic profiles of various LPS preparations and a commercial preparation of LPS purified from *Escherichia coli* (serotype 026-B6,Sigma) were performed by Ms Melanie Rutherford at the Department of Primary Industry, Water and the Environment, Launceston using SDS-PAGE with 12% polyacrylamide resolving gels. SDS-PAGE prior to electrotransfer to nitrocellulose for immunoblotting was performed under reducing conditions on 10% resolving gels using the protocol described in section 2.2.2. Western blots to analyse LPS preparations and the bacterin were carried out as described in section 2.2.5.

6.2.7 Statistical analyses

Data were analysed with a one-way analysis of variance (ANOVA) using the JMP 3.2.1 statistical package. ANOVA assumptions of data normality and homogeneity of variances were tested using the Shapiro-Wilk W test and Bartlett's test respectively. Nested analyses were used to ascertain differences due to time within treatments (i.e. sampling times of 7 and 21 days post booster). As found in previous experiments serum *V. harveyi* inhibitory activity data fulfilled neither assumptions of normality nor homogeneity of variances and was therefore analysed using the Kruskal-Wallis non-parametrical statistical test. In examining associations between specific antibody activity and *V. harveyi* inhibitory activity correlation coefficients were determined and tested for significance (Sokal and Rohlf, 1987). Significant differences were highlighted as multiple comparisons of means (Tukey-Kramer HSD test) were carried out. When examining differences in all parameters between aquaria and treatment groups at 7 and 21 days post booster, data were pooled if analyses had P values > 0.1. For all tests a significance level of $P < 0.05$ was adopted.

6.3 RESULTS

6.3.1 Lipopolysaccharide extraction

LPS extraction from *V. harveyi* was successful in terms of purity (Figure 6.1), however a yield of 1.5 mg was lower than expected. Yield is dependent on the state of the cells at time of harvest and is also influenced by the extraction technique. The technique used in this study was suitable for rough type (R-type) LPS. As the R-type LPS lack the O-antigen, the mobility patterns seen on PAGEs differ to those of the S-type LPS which has a marked multi-banding or laddering pattern. The LPS from *V. harveyi* was thought to be R-type due to the lack of laddering as seen on PAGEs of a crude LPS product extracted via proteinase K digestion of *V. harveyi* lysates.

6.3.2 Immune assays

There seemed to be a lack of macrophage activation by LPS, given that phagocytic indices of head kidney macrophage cell suspensions showed no significant difference ($P = 0.47$) 7 days after the booster (Figure 6.2). Unfortunately there are no results for this parameter at 21 days post booster due to bacterial contamination of the cell suspension. LPS did, however have an effect on lysozyme activity with all groups demonstrating significantly different levels in activity ($P < 0.001$) (Figure 6.3). Lysozyme activity was highest in the control group with both LPS- and bacterin-immunised groups showing depressed activity. There was however no difference within treatment groups at 7 or 21 days post booster for this parameter ($P = 0.36$), thus data were pooled at this level.

A specific humoral response was detected in both LPS and bacterin treatment groups with specific antibody activity detected being highly variable. When antibody responses of individual fish were examined (Figure 6.4A), five of the ten responded to LPS and five of nine to the bacterin (there was one mortality in the bacterin-immunised group), no antibody activity was detected in the control group. Statistical analysis showed no significant

differences within groups at 7 and 21 days post booster (Figure 6.4B), therefore the data were pooled. There was no significant difference in antibody activity between LPS- and bacterin-immunised groups. The bacteriostatic activity of the serum was also evident (Figure 6.5) and was significantly higher in the bacterin-immunised fish (P= 0.036). There was also some bacteriostatic activity in the control fish but this was due to 2 individual fish (Figure 6.5A). LPS-treated fish had lower levels of bacteriostatic activity but most did show some activity. Contrary to observations from a previous experiment there was no correlation between specific antibody activity and bacteriostatic activity of the serum (Figure 6.6). This lack of correlation between these parameters was noted when looking at the treatment groups both separately and combined.

Western blot analysis of LPS preparations and the bacterin (Figure 6.7) clearly show that both LPS- and bacterin-immunised fish have antibodies directed against *E coli* LPS, although there appears to be no reactivity against the proteinase K digestion product (crude *V. harveyi* LPS) or the bacterin.

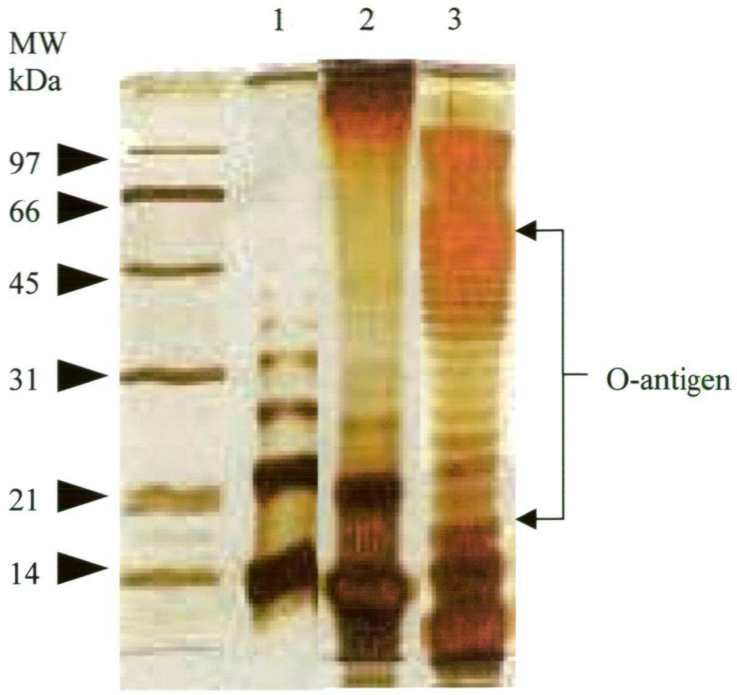

Figure 6.1 SDS-PAGE showing comparative purity of LPS extracted from *V. harveyi* (lane 1) by method of Sprott et al. (1994), LPS derived from proteinase K digestion of *V. harveyi* lysates (Hitchcock and Brown, 1983) (lane 2) and LPS from *E. coli* (Sigma) (lane 3) with the area of O-antigen indicated. The gel was silver stained.

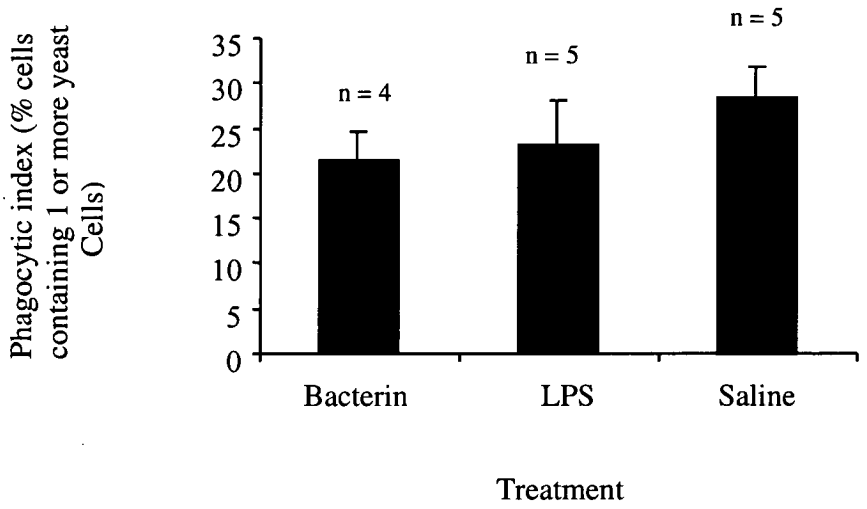

Figure 6.2 Phagocytic indices for barramundi anterior kidney macrophage suspensions 7 days after booster immunisation with LPS, *V. harveyi* bacterin or saline as a control, values are means + SE (no significant difference, P = 0.47).

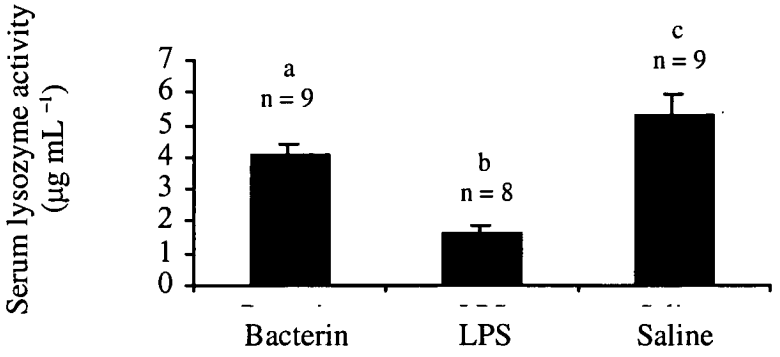

Figure 6.3 Serum lysozyme activity for barramundi after booster immunisations with LPS, *V. harveyi* bacterin or saline as a control. Data were pooled as there was no difference between 7 and 21 days post booster. Values are means + SE, disparate superscripts show significant differences between treatment groups (P < 0.001, Tukey-Kramer HSD test).

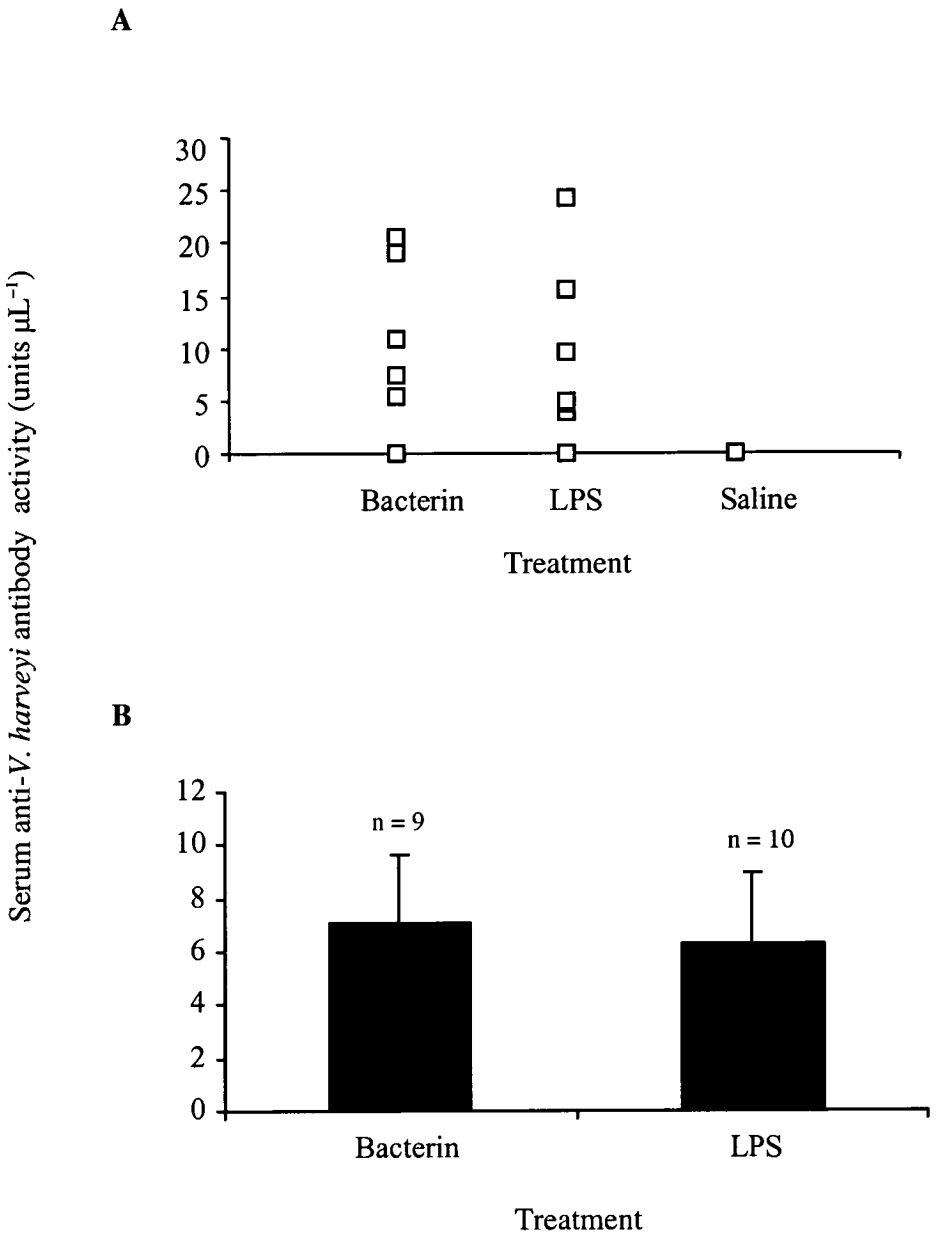

Figure 6.4 Specific anti- *V. harveyi* antibody activity of barramundi serum after booster immunisation with *V. harveyi* bacterin, LPS or saline. **A.** Individual fish within a treatment group. **B.** Activity of fish per treatment group, there was no activity detected in saline treated fish. Data were pooled as there was no difference between 7 and 21 days post booster. Values are means + SE (no significant difference).

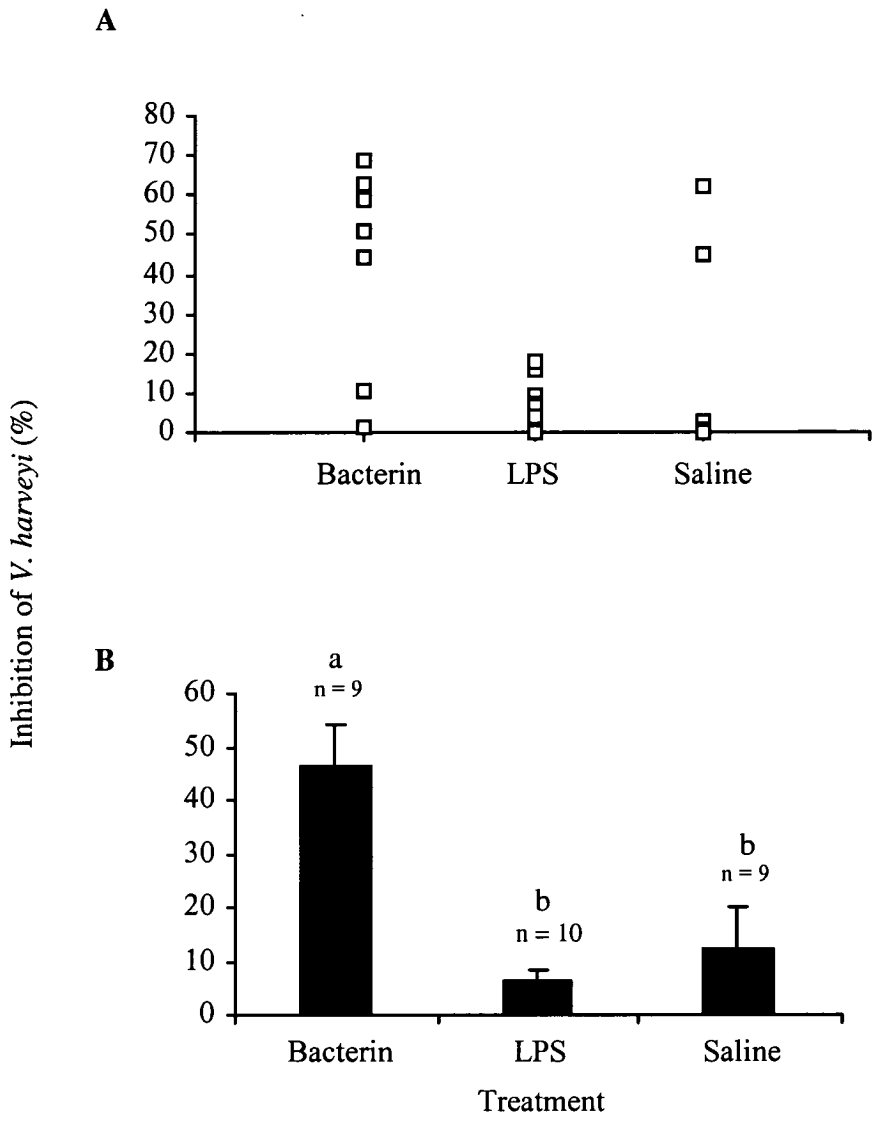

Figure 6.5 *V. harveyi* inhibitory activity of barramundi serum relative to pooled non-immune standard sera after booster immunisation with *V. harveyi* bacterin, LPS or saline. **A.** Individual fish within a treatment group. **B.** Fish per treatment group, data were pooled as there was no difference between 7 and 21 days post booster. Values are means + SE and disparate superscripts show significant differences between treatment groups (P = 0.03, Kruskal-Wallis test).

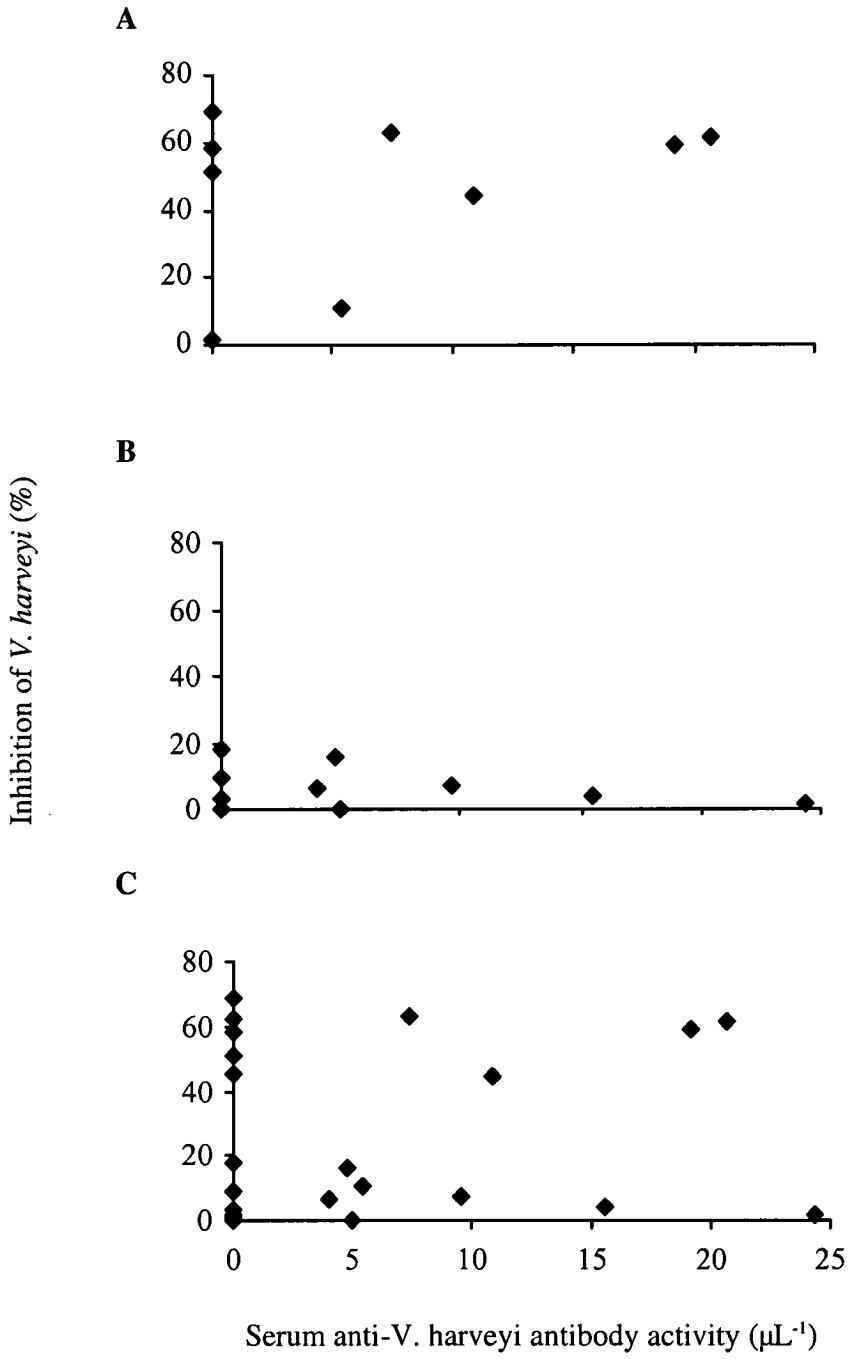

Figure 6.6 Relationship between anti-*V. harveyi* antibody activity and *V. harveyi* inhibitory activity (relative to pooled non-immune standard sera) of barramundi serum showing no significant correlation ($P > 0.05$). **A.** Bacterin immunised fish ($r = 0.285$, $n = 9$). **B.** LPS immunised fish ($r = 0.311$, $n = 10$). **C.** All treatment groups combined ($r = 0.195$, $n = 29$).

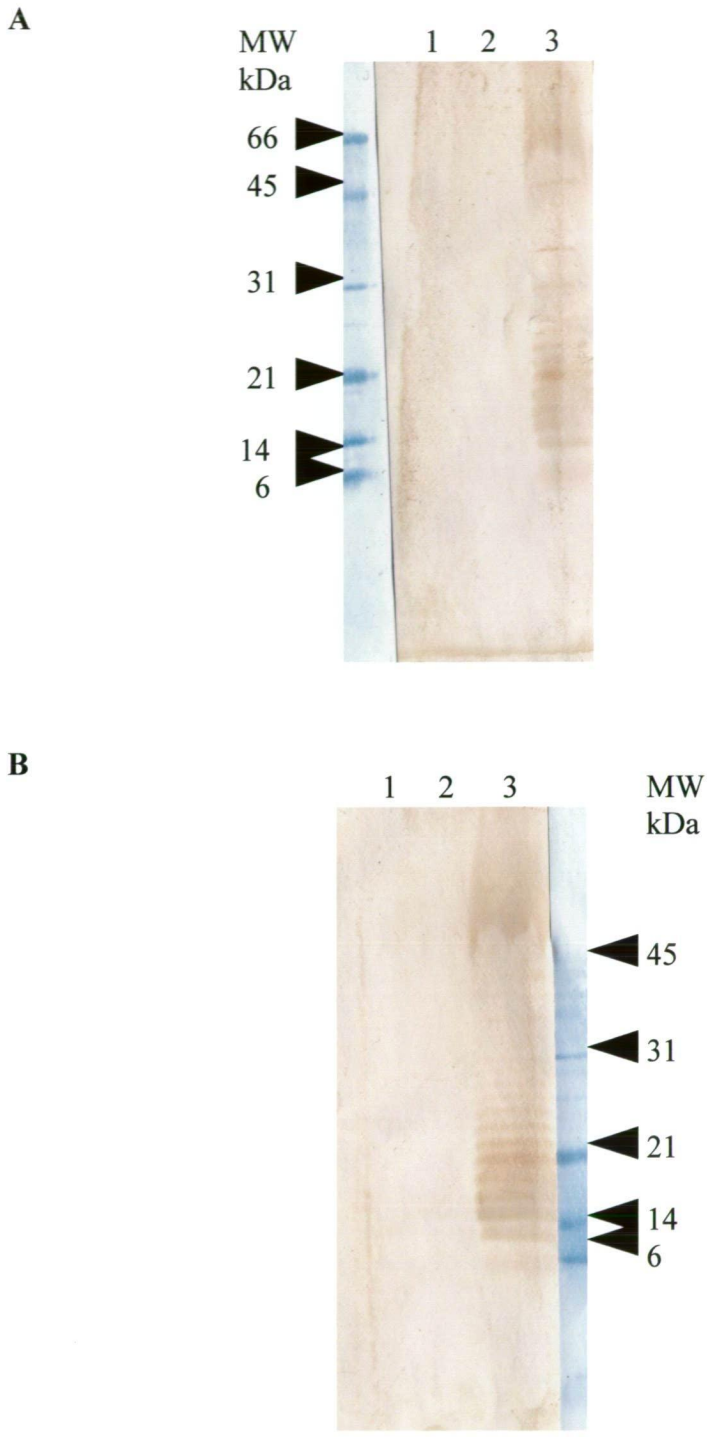

Figure 6.7 Western blot analysis *V. harveyi* LPS (proteinase K digest) (lane 1), *V. harveyi* sonicate (lane 2) and *E. coli* LPS (Sigma) (lane 3). **A**. Probed with pooled bacterin-immunised sera at 1:6 **B**. Probed with LPS-immunised pooled sera at 1:6

6.4 DISCUSSION

The results presented here demonstrate the ability of barramundi to respond immunologically to both a *V. harveyi* bacterin and LPS extracted from this bacterium. The extent of this response was varied and more pronounced in the specific humoral arm of the immune system than the non-specific parameters measured. LPS is very immunogenic in fish and is recognised as the predominant structure to which antibodies are directed when Atlantic salmon, *S. salar*, are immunised with cells of *V. salmonicida* and *V. anguillarum* (Bøgwald *et al.*, 1991). Whether this is true in this instance is not known as immunoblot analyses of both the bacterin and LPS were inconclusive, although both LPS- and bacterin-immunised fish had antibodies reactive with LPS from *E. coli*.

Variation in the antibody response to LPS may have been due to a number of reasons. First, the use of killed-*V. harveyi* cell sonicate as the coating antigen may have decreased the sensitivity of the ELISA. Second, individual variation between fish in the response may be expected as some fish may be non-responders. Third, there may be a dose effect i.e. it may have been too high or too low. Previously reported doses of LPS for fish vary widely, ranging from 20 ng kg^{-1} for coho salmon, *Oncorhynchus kisutch*, (Velji *et al.*, 1991), 10 mg kg^{-1} for turbot, *Scophthalmus maximus*, (Al-Harbi and Austin, 1992) and 50 mg kg^{-1} for Atlantic salmon, *S. salar*, (Dalmo and Bøgwald 1996), there is no published information on LPS administration to barramundi. Fourth, LPS may be more immunogenic in its natural state with its associated membrane and cytoplasmic proteins. Although many studies have used isolated LPS as an antigen (Velji *et al.*, 1991; Dalmo and Bøgwald 1996; Nakhla *et al.*, 1997), some studies have involved immunisation of fish with whole killed bacterial cells then used immunoblots and ELISAs to elucidate the roles of particular antigens like LPS (Hoel *et al.*, 1997). This latter method may be more pragmatic, at least when investigating antibody responses, as the purified LPS does not exist naturally and therefore assessments of its biological activity need to done with caution (Burrell, 1990). However, it would be difficult to appraise responses to particular antigens of other immune parameters without immunising with a purified antigen. With this in mind, the study was designed to compare

the response of extracted LPS with the whole cell bacterin in an attempt to see if LPS enhanced non-specific immunity in conjunction with eliciting an antibody response. The antibody response to the bacterin was also variable, with only five of the nine fish responding which was similar to the responding rate of LPS-immunised fish which was five from ten. Variable antibody response after immunisation is not an unknown observation in fish, non-responsiveness of B cells in the gut of rainbow trout, *O. mykiss* to key-limpet hemocyanin (KLH) has been reported though there was no concomitant systemic non-reponsiveness (Jones *et al.*, 1999). In carp, *C. carpio*, individual fish with a high and low antibody response are common enough for work to be performed on selection and propagation for further research (Wiegertjes *et al.*, 1995).

The *V. harveyi* inhibitory activity of the sera was highest for the bacterin-immunised fish which concurs with results noted in previous experiments reported in Chapters 4 and 5. Although two control fish also showed high bacteriostatic activity (Figure 6.5B), the activity noted in the LPS-immunised fish was more consistent. In this study no correlating relationship was found between anti-*V. harveyi* antibody activity in the serum and the bacterial inhibitory ability of the serum. This is contrary to what was reported Chapter 5. However, the serum antibody activities of IP bacterin-injected fish seen in that experiment were higher than reported here. This discrepancy in antibody activity maybe due to individual variation, assay variability or variation in the vaccine. Different batches of vaccine were used for each experiment and though they came from the same stock culture and were cultured under the same conditions, there may have been subtle differences caused by mutations within the original culture for example which may have altered immunogenicity. The consistently high serum bacteriostatic ability of bacterin-immunised fish suggests that this activity was a result of the immunisation. However, information on the bacteria-inhibiting mechanism of the immune serum has not been gained. The bacteriostatic assay has been performed with samples that were heat treated to inactivate complement (45°C for 10 min) and with untreated samples, with little difference being noted. It could be argued that in both untreated and heat treated samples complement may have already been inactivated in storage given its lability. If this was the case, then antibody may have been responsible for the bacteriostatic ability of the immune serum. Fish antibody

acts as an opsonin and can bind and agglutinate bacterial cells or neutralise antigens by blocking a critical function (Kaattari and Piganelli, 1996). The assay is based on the uptake of MTT by living bacterial cells and its subsequent reduction by mitochondrial enzymes to formazan, a process that may have been inhibited by opsonisation or agglutination. The other possibility, as discussed in Chapter 5, is that an unmeasured serum factor was responsible for the activity. In any case the apparent bacteriostatic activity of the immune serum suggests that vaccination with *V. harveyi* bacterin by IP injection may induce protection. In the absence of data from vaccination and challenge trials using the relevant *V. harveyi* isolate making such a link is tenuous.

LPS was demonstrated as an antigen in barramundi by ELISA, though its protective role remains unclear. The lower lysozyme activity noted in LPS-immunised fish relative to bacterin-immunised and control fish may or may not be a treatment effect. There are a number of reports where lysozyme activity of the serum has been shown to increase after immunisation with various preparations (Rainger and Rowley, 1993; Chen *et al.*, 1998). However, Ainsworth *et al.* (1995) found no detectable trends. Increase in lysozyme activity in plasma seems a typical response of rainbow trout under acute stress (Fevolden and Roed, 1993; Demers and Bayne, 1997), it has even been mooted as a possible replacement for cortisol as a stress indicator in fish (Fevolden and Roed, 1993). Whether fish in the bacterin and control groups were more stressed than LPS-immunised fish is not known though the holding facilities and handling procedures were uniform. A group naïve to all treatments, including handling, would have been an advantage in this experiment to ascertain base levels of lysozyme activity in serum. Such a group was included in the previous experiment (Chapter 5), and there was little difference noted between naïve, control and immunised fish.

The LPS extraction method resulted in a yield lower than expected, given the culture volumes used. This may stem from the method used and/or the physiological state of the bacterial cultures. The extraction method suitable for R-type LPS was chosen based on the banding patterns of a crude LPS preparation from *V. harveyi*.. The lack of laddering on PAGEs suggested that the LPS was R-type. The laddering appearance on gels results from the region of LPS comprising the outer oligosaccharide side chain or O-antigen. This side

structure is in fact a heterogeneous mixture of oligosaccharides of various chain lengths and subsequently different mobilites on gels. R-type LPS lack this outer side chain. There seems to be little or no information on LPS of *V. harveyi* in the literature and the structure varies widely between different strains and species of Gram negative bacteria (Sprott *et al.*, 1994). What may cloud the issue even more is that multiple forms of LPS may be produced by a single strain of a bacterium depending on culture conditions such as temperature, cell densities and nutrition. As pointed out by Sprott *et al.* (1994), the heterogeneity of LPS was not fully appreciated when extraction methods were developed, therefore yields from any particular method may be equally varied. Whether or not the heterogeneity of LPS impacts on its immunogenicity is not known.

Regarding the specific antibody responses, the evidence from immunblots using *E. coli* LPS, the crude *V. harveyi* LPS from the proteinase kinase digest and, the bacterial sonicate suggests that antibodies elicited in both bacterin-and LPS-immunised groups may be directed against LPS and that there are possibly shared epitopes between both *E. coli* and *V. harveyi* LPS or, that individuals had a some exposure to environmental Gram negative bacteria and LPS. The cross-reactivity with the *E. coli* LPS is thought likely because in a subsequent experiment in our laboratory barramundi immunised with a PBS placebo were found to have antibodies reactive with the *V. harveyi* sonicate in ELISAs, and further that these sera were reactive with *E. coli* LPS as determined by Western blot (Bridle, 2000). The lack of any obvious reactivity with *V. harveyi* sonicate or the proteinase K digest of immune sera in Western blots may imply that there is relatively insufficient LPS in these preparations to be detected by this method as opposed to the highly-purified commercially prepared *E. coli* LPS. However, reactivity of immune sera with the bacterial sonicate detected by ELISA possibly highlights the greater sensitivity of ELISA compared to western blot. The disparity between *V. harveyi* inhibitory activity of non-immunised and immunised sera indicates that even though both sera have apparent antibody activity, as measured by ELISA, the antibodies in the non-immune sera may be less relevant. Explanations as to why placebo-immunised fish displayed *V. harveyi* inhibitory activity and no specific antibody activity are difficult to find. Certainly, it may be due to individual variation given the lack of extensive replication in this experiment.

CHAPTER 7

GENERAL DISCUSSION

Overall, the experiments described in this thesis clearly indicate *V. harveyi* bacterin to be a good vaccine candidate for barramundi. The results consistently demonstrate the ability of the bacterin to elicit a systemic immune response in barramundi. Moreover, sera with high specific antibody activity also displayed high antibacterial activity, although an association between the two was not proven. Clearly the next step is to evaluate the bacterin using vaccination/challenge experiments. As alluded to in Chapter 1 studies described in this thesis formed only one part of a collaborative effort to develop and evaluate a vaccine and unfortunately data from commercial vaccination/challenge trials are not yet available.

Although it cannot be inferred from the present study that induction of an immune response is an indication of effective vaccination, there have been studies that related specific antibody to protection from disease. Joosten *et al.* (1995) and Joosten *et al.* (1996) found a positive correlation between specific antibody and protection using *V. anguillarum* bacterin in carp. Similarly, a positive association has been observed in the humoral response and protection after IP immunisation against furunculosis in Atlantic salmon (Midtlyng *et al.*, 1996a; Midtlyng *et al.*, 1996b; Bricknell *et al.*, 1999), and after IP immunisation against an atypical *A. salmonicida* strain (Gudmundsdóttir *et al.*, 1997). However, correlations between specific antibody and protection do not always occur (Smith, 1988) and this lack of correlation underlines the requirement to identify antigenic determinants within a prospective vaccine. A bacterin is a complex mixture of whole cells, extracellular and intracellular products some of which will be virulence factors and all may vary in immunogenicity. For example, a furunculosis vaccine prepared from extracellular products of *A. salmonicida* elicited systemic antibody in rainbow trout to only four of the fourteen proteins present (Hastings and Ellis, 1988). This also highlights the need to investigate the specific immunity to particular antigens as potentially protective antigens may or may not be immunogenic in the target fish, i.e. some epitopes may be immunodominant (Vallejo *et al.*, 1992).

To be protective antibodies need to exert secondary effector functions other than the primary function of binding antigen (Roitt *et al.*, 1996). These functions need to be neutralising in some way: they need to block some critical function such as adhesion by a pathogen, agglutinate or opsonise it to promote phagocytosis, neutralise any extracellular toxins produced as a result of infection or, activate complement to facilitate elimination of the microbe (Kaattari and Piganelli, 1996). In the case of *V. anguillarum* vaccines protection has been unequivocally linked to specific antibody by passive immunisation experiments where immune sera were derived from injection immunisation (Harrell *et al.*, 1975) and immersion immunisation (Viele *et al.*, 1980). However, protection against vibriosis has also been noted after hyperosmotic infiltration (HI) and IP immunisations with little or no detectable antibody (Croy and Amend, 1977), although in many instances attempts to detect antibody used agglutination assays. Lack of detectable antibody indicates that factors other than systemic antibody may also be involved in protection, or that agglutinating antibody detection methods may be insensitive. Conversely, protection against vibriosis in the presence of specific antibody was seen after bacterin delivery via HI and IP injection (Antipa and Amend, 1977) and spray immunisation (Gould *et al.*, 1978). So, in the case of vaccine-induced protection against disease from *V. anguillarum* small amounts of systemic antibody may be sufficient. Unfortunately, in the example cited above of successful passive immunisation using immune sera derived from immersion (Viele *et al.*, 1980) antibody levels in the immune sera were not measured. However, in another successful passive immunisation experiment where plasma immunoglobulins, derived after immunisation with *V. ordalii* LPS, were injected into coho salmon, no bacterial cell agglutinating antibody was found (Velji *et al.*, 1991). Boesen et al. (1999a) found that rainbow trout serum was still bactericidal against *V. anguillarum* when diluted to the extent that it no longer agglutinated the bacterium provided a complement source was added. This supports the notion that small amounts of antibody undetectable by agglutination assays are sufficient for protection.

An additional explanation for protection in the apparent absence of antibody relates to the routes of immunisation. For example *V. anguillarum* vaccines are routinely delivered via IP injection and immersion (Lillehaug, 1989b) and, as noted previously, are generally

successful regardless of the level of systemic antibody. Aside from the cellular mechanisms that may be involved, this may indicate that protection occurs at the mucosal or systemic level depending on the route of immunisation. Thus to clarify the situation it seems imperative to be able to detect mucosal antibody.

In the experiments described in this thesis systemic antibody after bacterin delivery by immersion was detected although at lower levels than those seen after IP administration. Production of systemic antibody after immersion immunisation is not surprising given antigen uptake occurs across skin (Ototake *et al.*, 1996; Moore *et al.*, 1998) and the gill tissue (Smith, 1982; Zapata *et al.*, 1987) and appears in the anterior kidney and spleen after a few hours (Smith, 1988). However, antibody was undetectable in barramundi mucus and, as discussed in Chapter 4, is possibly because the detection methods were not sensitive enough. Detection of antibody in mucus samples by ELISA has been reported after enteric delivery of a reference antigen, human gamma globulin (HGG), to tilapia (Jenkins *et al.*, 1994) and, after IP and oral delivery of *Edwardsiella ictaluri* antigen to channel catfish (Ainsworth *et al.*, 1995), although at very much lower levels than those seen in serum. It seems unlikely that there was no antibody present in barramundi mucus given published data on existence and elicitation of mucosal Ig in numerous species (Table 1.1).

Elevated humoral responses observed after secondary (booster) IP immunisations in all experiments suggested a piscine equivalent of an anamnestic response. The experiment described in Chapter 4 showed a three-fold increase in antibody activity after booster immunisations. In this experiment the use of oil-based adjuvant in both primary and secondary immunisations may have been responsible for the secondary rise in antibody activity. Previous reports of a second peak in antibody titer (Lamers, 1986) and antibody-secreting cells in the head kidney (Davidson *et al.*, 1993) over time after only a primary immunisation have been attributed to an adjuvant effect. However, heightened antibody responses after IP booster were found in a subsequent experiment (Chapters 5) where no adjuvant was used. One of the limitations of the design of each of the experiments was that no groups of fish were given a primary immunisation only. Such a group may have clarified observations that elevated humoral responses were actual secondary responses and not a

result of the kinetics of the response. Thus results need to be interpreted in that context. However, if the peak antibody titer after primary immunisation occurred within the period before the secondary immunisation then subsequent increases could be attributed to the secondary immunisation. In the present experiments, the time between primary and secondary immunisations was 21 days and it is possible that a primary peak occurred within that time. Previous studies indicate that peaks in antibody levels in fish occur within a range of time frames but these are probably dependent on the nature of the antigen, the dose, administration route and the water temperature. The serum antibody response of channel catfish to a primary IP immunisation with *E. ictaluri* bacterin showed a peak after 21 and 28 days for two different experiments with the disparity attributed to temperature differences (Vinitnantharat and Plumb, 1992). Temperature has been shown to influence the magnitude and kinetics of an antibody response in hybrid sunshine bass (*Morone chrysops* X *Morone saxatilis*) with lower than optimal temperatures for the hybrid slowing the rate and decreasing the magnitude of the response to *A. salmonicida* (Hrubec *et al.*, 1996). Serum antibody peaks in rainbow trout administered *Y. ruckeri* bacterin via IP injection were found after 28 days (Verlhac *et al.*, 1996), however in this example the peak may have been earlier or even later as fish were only sampled every 14 days. Similarly in rainbow trout, after IP injection with bivalent vaccine made up of *V. anguillarum* and *A. salmonicida* antigens, Lúnden *et al.* (1998) found antibody against *V. anguillarum* increasing 12 weeks post vaccination but a peak after 28 days for antibody against *A. salmonicida.* Secombes *et al.* (1982) found peak antibody in carp serum 35-42 days after IP injection with HGG then a decline. Given these time frames for antibody responses it is feasible that in my study the antibody levels were at least near peak levels within 21 days of the primary immunisation. However, without sufficient data on the systemic antibody response of barramundi to bacterin it is difficult to hypothesise on the kinetics of the response.

An elevated antibody response was not seen in groups immersed nor anally intubated with bacterin. The kinetics of the humoral response may also be a factor in these cases, fish were sampled 10 and 21 days after booster immunisations (immersion and anal intubation) and it is possible that the antibody response had not reached its peak in that time frame or had reached a peak then declined. Jenkins *et al.* (1994) found a peak serum antibody response

14-21 days after anal and oral delivery of HGG then a decline. The kinetics of the systemic response will vary with the administration method as shown by Palm *et al.* (1998). In experiments using a *V. anguillarum* bacterin administered to rainbow trout these authors reported serum antibody peaks 17 days after initial immersion delivery, 24-31 days after IP injection and virtually no antibody titers within 42 days after oral delivery. In the same experiment peaks after secondary immunisations were after 10 days for orally-treated fish and 17 days for both immersion and IP injected groups (Palm *et al.*, 1998).

Immunological memory in fish is yet to be fully elucidated. If, as Kaattari (1994) proposes, the definition for a piscine memory response is based on a distinction in form and function leading to enhanced immune reactivity, then a faster antibody response of a higher magnitude after secondary exposure partially fits the definition. In the present experiment, where bacterin was delivered via IP injection without adjuvant (Chapter 5), there was a four-fold increase in antibody activity after secondary administration. This observation at least fits one of the criteria for immunological memory in fish. Thus, from the data presented here it can be concluded that there are indications of an anamnestic response in barramundi, further studies investigating the kinetics of antibody responses in barramundi to a bacterin are required for confirmation.

In comparison to antibody activities found in subsequent experiments after IP immunisation, those found in Chapter 4 are very high with activity units in the order of 200-300 compared to less than 20. Reasons for this disparity may be due to the adjuvant effect and/or because a bacterin derived from a different strain was used exclusively in this first experiment and therefore reagents used in the ELISA varied. It should be pointed out that a degree of repetition in some of the experiments described in the thesis was unavoidable. During the course of the study two different strains of *V. harveyi* were used because a more virulent strain was identified after initiation of the project. These strains were not previously characterised serologically and unfortunately attempts to do so via Western blot were not successful. It seems reasonable to assume that increased virulence of one strain may be due to some factor not present in the less virulent strain. Olivier *et al.* (1985) found variable immunogenicity from virulent and avirulent strains of *A. salmonicida* in coho salmon,

Oncorhynchus kisutch. It was therefore necessary to use the most virulent strain of *V. harveyi* for subsequent experiments.

Observations of a systemic humoral response after bacterin delivery via the mucosa suggest that both immersion and oral delivery methods have potential. As with bacterin vaccines against other vibrio pathogens such as *V. anguillarum*, *V. ordalii* and *V. salmonicida* where immersion delivery is effective (Newman, 1993), indications are that the same may well be true for vaccines against *V. harveyi*. A pertinent point is the lack of domestication of barramundi and the need to limit the stress involved in vaccination. A systemic response elicited after bacterin delivery to the GALT of barramundi via anal intubation suggests that oral delivery methods are worth investigation. This has been vigorous area of research as vaccine administration perorally is seen as the only stress-free method and is the most desirable by aquaculturists (Ellis, 1988b). Problems associated with antigen degradation in the gut can be ameliorated by antigen encapsulation, and current research in this area has shown promising results (e.g. Joosten *et al.*, 1995; O'Donnell *et al.*, 1996; Azad *et al.*, 1999).

Detection of anti-*V. harveyi* antibody activity in some non-immunised fish from two of the experiments complicated interpretation of the ELISA results. As argued in Chapter 5 this was thought to be due to cross-reactivities of non-immune sera with some bacterial antigens, or previous exposure to *V. harveyi* and not to inadequacies of the assay. A similar observation made in cod, *Gadus morhua*, was attributed to natural antibodies in the fish cross-reacting with bacterial antigens which were not linked to LPS (Espelid *et al.*, 1991). Immunoblots using cross-reactive sera from non-immunised barramundi in a related experiment carried out in our laboratory (Bridle, 2000) did indicate a role for LPS. These immunoblots showed cross-reactivity with LPS from both *Escherichia coli* and *V. harveyi*. An equally plausible explanation for antibody activity in non-immunised fish is the previous exposure theory. The barramundi used in these experiments were hatchery-reared in seawater and it is possible that they were exposed to bacteria from the genus *Vibrio*. Given that *V. harveyi* is a common water-borne organism (Austin and Austin, 1993), this may have been the case with some of the barramundi used in these experiments. In any case, it is

believed that the results of the ELISA accurately reflect the humoral responses of barramundi to the bacterin. Immune and non-immune control sera were included on every plate with consistent results, and there was a trend for higher activity in those experimental fish that were bacterin-immunised. Moreover, there was a far more consistent response in the bacterin-immunised fish as shown in individual response data (Figures 4.7A and 5.4A).

The fact that sera with high anti-*V. harveyi* antibody activity consistently displayed high inhibitory activity against the bacterium suggests that such sera may confer protection against vibriosis caused by *V. harveyi*. Results of bacteriostatic assays presented here appear contrary to the possibility of low antibody levels being protective. High *V. harveyi* inhibitory activity was only seen in sera of high antibody titer while sera of low or no antibody titer as well as all mucus samples showed little or no inhibitory activity. As reported previously, protection after immunisation has been demonstrated with little or no serum antibody present (Croy and Amend, 1977; Baba *et al.*, 1988; Thorburn and Jansson, 1988). A major factor of the present observations is the sensitivity of the bacterial inhibitory assay and it is possible that numbers of bacterial cells used in the assay do not reflect cell numbers encountered by the primed immune system in the early stages of infection in fish. Although, the only definite way to demonstrate vaccine effectiveness is a significant reduction in mortality on challenge with the pathogen, a sensitive bacterial inhibitory assay may have practical applications. For example use of this relatively simple and inexpensive assay may be helpful in monitoring *in vivo* immunogenicity in various vaccine batches. There is also the potential to adapt the assay for use with other bacterial pathogens.

Lipopolysaccharide extracted from *V. harveyi* was shown to be immunogenic in barramundi. Antibody levels elicited after LPS immunisation were as high as those found in bacterin-immunised barramundi. However, the antibacterial activity of the sera from the LPS-immunised group was much lower. As mentioned previously, this observation may relate to the sensitivities of the bacteriostatic assay and that numbers of bacteria used in the assay may overwhelm antibody levels that may be effective *in vivo*. Performance of the antibacterial assay with a range of concentrations of bacterial cells may have found a point where LPS-immunised sera did display some antibacterial activity. Unfortunately it was not

possible to repeat the assay due to the small quantities of sera obtained from experimental fish and volumes required. An alternative suggestion is that the role of LPS as a protective immunogen may be due to mechanisms other than LPS-specific antibody. Immune responses to endotoxin, which is the biological form of LPS and includes associated membrane proteins, can result in specific antibody production but, can also occur via the cell-mediated arm where stimulated cells would facilitate elimination of microbes containing endotoxin (Morrison and Ryan, 1979). The role of LPS as a protective antigen has been demonstrated in vaccines against some bacterial fish pathogens, including *V. anguillarum, V. salmonicida* and *Y. ruckeri* (Press and Lillehaug, 1995) although the mechanisms of protection are unclear. However, the protective role of anti-LPS antibodies was shown in a passive immunisation experiment using sera from coho salmon immunised with LPS extracted form *V. ordalii* (Velji *et al.*, 1991). In this experiment naïve fish were protected after immunisation with partially purified plasma immunoglobulins derived from IP- and immersion-immunised fish however, when these plasma immunoglobulins were absorbed against *V. ordalii* cells prior to injection into naïve fish no protection was transferred (Velji *et al.*, 1991). A third explanation for a lack of antibacterial activity in LPS-immunised barramundi is that LPS is not a protective antigen of *Vibrio harveyi.* This theory would need to be based on the assumption that the bacteriostatic assay does in fact give an indication to the protective nature of the bacterin in the absence of vaccination/challenge data.

In other animals the protective role of LPS is controversial with conflicting results from passive and active immunisation experiments (Muniandy *et al.*, 1998). In fish results are also variable, Baba *et al.* (1988) found better protection in carp from *A. hydrophila* infection with a crude preparation of LPS than with a bacterin. These authors suggested the lack of bacteria-agglutinating antibody indicated that protection was not due to humoral immunity. The dominant antigen in Gram negative bacterial vaccines is LPS in that the majority of antibody is directed against the O-antigens (i.e. the repeating oligosaccharide units) (Morrison and Ryan, 1979). However, it should be noted that oligosaccharide units of LPS are extremely heterogenous within species and strains (Morrison and Ryan, 1979) which may impact on the protective nature of LPS as an antigen. Using a *V. anguillarum* and

rainbow trout model, it has been suggested that there is a link between the length of these units and the bactericidal activity of fish sera (Boesen *et al.*, 1999b). These authors found a decrease in the high molecular weight (HMW) components of *V. anguillarum* O-antigen when glucose was added to the culture medium and further that this decrease corresponded with an increase in susceptibility to killing by serum. It was postulated that existence of HMW O-antigens on *V. anguillarum* cells may sterically hinder complement access to the cytoplasmic membrane (Boesen *et al.*, 1999b). Analysis of LPS from *V. harveyi* via SDS-PAGE (Figure 6.1) showed a distinct lack of banding in the HMW region of the gel indicating the lack of an extensive range of O-antigens. Based on the logic of Boesen *et al.* (1999b) this may indicate a susceptibility of *V. harveyi* to action by serum which agrees with my findings of *V. harveyi* inhibitory activity of IP-immunised barramundi sera. Expression of O-antigen seems to be dependent on culture conditions (Boesen *et al.*, 1999) and in laboratory cultures may be governed by mutations (Reeve, 1995). The implications of these findings for *V. harveyi* culture and vaccine development is not known, more work is required to test if these observations are applicable to environmental *V. harveyi* .

The fact that LPS is the protective antigen for some fish pathogens poses an interesting question: how does it confer protection over time given that it is a classical T-independent antigen and therefore elicits a poor memory response? Retention of LPS within lymphoid tissue in salmonids has been shown to occur for up to 16 weeks (Press *et al.*, 1996), and this persistence would enable constant exposure to B cells and subsequent antibody production. The concept of LPS inducing poor immunological memory seems challenged by observations in mice that a sub-immunogenic priming dose of LPS can lead to either immunological memory or unresponsiveness, depending on the serotype, although the mechanisms involved are not known (Elkins *et al.*, 1989). Further in catfish, LPS was shown to act as a mitogen in sIg^- lymphocytes (T cells) (Sizemore *et al.*, 1984). Clearly, more work is required to fully elucidate the complexities involved in the interaction of LPS and the immune system. Although from a practical perspective the fact that bacterial vaccines, where LPS is the protective antigen, provide protection would indicate that the memory component has been elicited.

Another aspect of the of data presented in this thesis was the development of reagents to monitor specific humoral immunity in barramundi. These reagents have been used in experiments that investigated the adjuvant effect of recombinant interleukin 1ß in barramundi (Bridle, 2000). A further application could be a more detailed examination of B cells and their expression given that some of the reagents seem capable of detecting B cells from populations of leucocytes. This apparent ability to bind B cells is fortuitous as mammalian polyclonal antibodies raised against Ig from fish are generally found to be reactive with all the fish lymphocytes (Miller *et al.*, 1998).

In conclusion, the work described in this thesis will contribute to the development of an efficacious vaccine against vibriosis caused by *V. harveyi* infection. The work also forms part of the growing body of information on barramundi immunity which will also be applicable in the development of vaccines against other pathogens. The results indicate the direction that further research should take, both to fully elucidate barramundi immunity and develop immunoprophylaxis. Evaluation of the bacterin by vaccination/challenge trials is still a priority. The phenomenon of immunological memory remains controversial for barramundi as it does for other teleosts. Although there are indications of memory induction after IP immunisation with a bacterin in barramundi immunological memory should continue to be an on-going area of research. Similarly, the protective role of LPS of *V. harveyi* is unclear and *in vitro* and *in vivo* growth conditions influencing expression of O-antigens need definition. Full elucidation of mucosal immunity should enhance the prospects of success in delivery of vaccines by methods other than injection. Elicitation of systemic antibody after bacterin administration by immersion and anal intubation indicates that these two immunisation methods are worth pursuing.

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