Comparison of passive and active immunisation of fish against microbial diseases with consideration of the mechanisms involved

by:

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Abstract

Passive immunisation of fish was carried out to determine whether anti-*Vibrio anguillarum* antibodies (AVA), anti-*Streptococcus* sp. antibodies (ASA), and anti-*Paramoeba* sp. antibodies (APA) raised in sheep, rabbits and rainbow trout (*Oncorhynchus mykiss*) were persistent and biologically active in rainbow trout. Results of passive immunisation were compared with results of active immunisation in which fish were immunised by immersion in or injection with formalin-killed cells. Assessments of passive and active immunisation were undertaken concurrently for up to three months in order to demonstrate their relative efficacies and, especially, to evaluate the practical potential of passive immunisation.

Sheep and rabbit antibodies were detected in fish sera by enzyme-linked immunosorbent assay (ELISA) for up to 70 days after intraperitoneal (i.p.) injection. The relative percent survival (RPS) of fish challenged with virulent *V. anguillarum* after an i.p. injection (0.1 ml 100 g^{-1} fish body weight) of sheep, rabbit or fish AVA was 93.3, 86.6, 40% at one month; 25, 18.7, 12.5% at two months and 13.3, 4.1, 9.0% at three months post-immunisation (p.i.) respectively. In order to obtain equivalent protection to undiluted fish serum, rabbit and sheep sera had to be diluted 1: 8 and 1: 50 respectively. Protection conferred by immune sera was shown to be due to the specific antibodies alone. Also inactivation of complement from sera had no effect on the potency of either immune or non-immune sera. Affinity purified sheep and rabbit AVA sera were demonstrated to have equivalent protective potential as whole antisera in rainbow trout. Fish actively immunised by immersion showed RPS of 86.6, 93, and 81.8% after one, two and three months p.i. respectively in the same trials. Fish immune responses to sheep and rabbit antisera were determined (using specific ELISA) with the greater response being to the rabbit serum.

Rainbow trout given sheep AVA conjugated to LTB (the GM-1-binding subunit of *Escherichia coli* heat-labile toxin) orally had a RPS of 37.5% at 15 days and a RPS of 27% after one month post-immunisation. In contrast, sheep AVA conjugated to TraT (an internal membrane of *E. coli*) and Quil-A had RPSs of 18.7 and 6.2% after fifteen days and 13.3 and 0% after one month p.i. respectively.

The relative percent survival of rainbow trout challenged with virulent *Streptococcus* sp. after an i.p. injection (0.1 ml 100 g^{-1} fish body weight) of sheep, rabbit or fish anti-*Streptococcus* sp. antibodies was 88.8, 50, 0.0% after one month ; 33.3, 6.8, 6.8% after two months and 13.3, 0, 6.6% after three months p.i. respectively. Fish immunised actively had RPS of 88.8 and 11.1% after one month, 38.1 and 4.7% after two months 36 and 0.0% after three months p.i. for the injection and immersion routes respectively. Thus, passive immunisation shows potential as a therapeutic and prophylactic against streptococcosis as it gives similar results to active intraperitoneal vaccination and would be expected to provide immediate protection.

In one trial Atlantic salmon immunised i.p. with *Paramoeba* vaccines showed marked humoral responses. In another trial, Atlantic salmon were immunised passively with an i.p. injection (0.1 ml 100 g^{-1} fish body weight) of sheep APA. Immunised fish (both actively and passively) were exposed to a natural infection (by cohabitation with infected Atlantic salmon) one month post-immunisation. Transmission of the disease was successful. No unequivocal protection was demonstrated in any of the immunised fish in this experimental challenge trial. Moreover, local antibody against *Paramoeba* sp. in gill mucus of experimentally infected Atlantic salmon was not detected (by ELISA).

It is shown in this study that passive immunisation has significant potential in disease prevention when given to fish at strategic times, especially in the face of an outbreak when there is not enough time for an active immune response. Thus, there should be a future for the use of mammalian hyperimmune sera against fish diseases, in particular, those for which useful immunity by active immunisation is not available.

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General introduction

There are a limited number of vaccines for active immunisation of fish against important systemic diseases that cause serious economic loss in fish culture. Even these vaccines have limitations in their use. There is evidence that antisera produced in mammals could be useful in controlling important diseases of cultured fish if given at strategic points in the growth cycle. Harrell *et al.* (1975) showed that rabbit or trout antiserum made against *Vibrio anguillarum* appeared protective against experimental vibriosis for more than two months. In another experiment, brook trout were immunised passively by intraperitoneal injection against *Aeromonas salmonicida* and infected with a virulent strain of *A. salmonicida* at the time of immunisation; passive immunisation was concluded to be efficacious in preventing the disease (Marquis and Lallier, 1989). Protection of fish was achieved by injection of an anti-*A. salmonicida* hyperimmune serum (purified immunoglobulin G), and this lasted from 110 to 150 days when applied either prophylactically or therapeutically (Turgeon and Elazhary, 1992). These promising results encouraged the author to look at more critically at passive immunisation using a variety of systems.

It is therefore worthwhile to introduce passive immunisation (hyperimmune sera from homeotherms and poikilotherms when given to poikilotherms) as an effective method of protection against diseases that are economically important to cultured fish. Additionally, passive immunisation studies can provide important information about immunogenic proteins, requirement for multiple vaccine strains, pathogenesis of microorganisms, and titre of antibody required for protection (LaPatra *et al.*, 1994).

The principal aim of this research was to demonstrate the value of this approach using an organism, *V. anguillarum* about which much is known. The techniques developed were also applied to a specific *Steptococcus* sp. of rainbow trout which at present can not be controlled by vaccination (Carson and Munday, 1990), and a *Paramoeba* sp. of salmonid fish. In this study, the aims were to investigate passive immunisation of rainbow trout with hyperimmune sera of mammals and fish and demonstrate the longevity and protectiveness of these antibodies for up to three months in comparison with active immunisation, and evaluate the therapeutic and prophylactic efficacies in order to demonstrate the practical implications and feasibility of this vaccination method. A sensitive enzyme linked immunosorbent assay was employed to detect longevity of conferred antibodies about which little such data has been reported previously. Unlike antimicrobial substances that last 10 days or less, hyperimmune sera provide protection for 22 to 359 days against furunculosis (Turgeon and Elazhary, 1992). As they are immunoglobulins, there is no danger of drug resistance development, medication residue, or the need for withdrawal times (Turgeon and Elazhary, 1992). However there is likely to be an active immune response against the foreign immunoglobulins which may limit their persistence and efficacy.

General aims of this study:

Now that commercial wild fish stocks are overexploited and with the potential for extensive disease outbreaks in the developing aquaculture industry all over the world, there may be a difficulty maintaining current yields of fish proteins. Vibriosis, streptococcosis and paramoebiasis can be financially catastrophic and a major determinant of fish farms viability. Therefore, the need for disease control and management is obvious. It would be of great significance to provide effective methods of immunisation and introduce new vaccines to aquaculture, so in this project, a series of experiments was performed to assess how long the passively transferred antisera persist and how effective passive immunisation can be to protect fish against vibriosis, streptococcosis and paramoebiasis.

A small number of studies used an agglutination test for detecting the injected antibodies. However, in this study the rate of clearance of these antibodies was carried out using ELISA. This project addresses the following objectives in relation to vibriosis, streptococcosis and paramoebiasis with a view to providing practical, alternative disease control strategies.

I To passively immunise fish at serious risk of disease, when there is insufficient time to immunise them actively.

II To compare the efficacy of passive and active immunisation.

III To immunise fish orally using purified immunoglobulins conjugated to different vaccine carriers.

IV To provide non-chemical therapies for these infectious diseases.

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CHAPTER 1.0

GENERAL LITERATURE REVIEW

1.1 Passive and active immunisation of fish:

1.1.1 Passive immunisation:

Several studies have been conducted on passive immunisation including the use of fish or mammalian antisera. Some of these reports indicate acceptable levels of protection (Harrell, 1975; Olivier *et al.,* 1985; Turgeon and Elazhary, 1992). However, very few attempts have been made to evaluate the longevity of passively transferred antisera, the level of protection and, also, the feasibility of this method of immunisation in commercial fish industries. By using pre-prepared specific antiserum, fish become immune several hours after the administration of immune serum in contrast to several weeks required for active immunisation to become effective.

Passive immunisation has been used mainly against furunculosis in order to study antigenic components of the virulent *Aeromonas salmonicida* for producing an effective vaccine and to a lesser extent against vibriosis, edwardsiellosis and some other fish diseases as summarised in Table 1.1.1.

Early results using passive immunisation against furunculosis were not encouraging, however, additional research proved it to be effective. Passive protection against *A. salmonicida* was produced in coho salmon (*Oncorhynchus kisutch*) using serum from rainbow trout (*Oncorhynchus mykiss*) containing antibodies produced following intraperitoneal (i.p.) vaccination (Spence *et al.,* 1965).

In another study, rabbit immunoglobulins produced against *A. salmonicida* did not provide protective immunity in salmonids, but Atlantic salmon (*Salmo salar*) passively immunised with antiserum prepared in a closely related salmonid were resistant to challenge (Cipriano, 1981). Also, in another successful experiment, brook trout (*Salvelinus fontinalis*) passively immunised with antiserum from rainbow trout prepared against virulent *A. salmonicida* were protected (Cipriano, 1982).

McCarthy *et al.* (1983) studied protection against *A. salmonicida* by passive immunisation and showed that antiserum to a rough, virulent strain of *A. salmonicida* (AS-IR) prepared in rabbits passively protected sockeye salmon, (*Oncorhynchus nerka*) against a virulent challenge, while antisera to a boiled AS-IR, and an avirulent smooth strain of the same isolate (AS-IS) were not protective. It was shown that the AS-IR cells contained an additional antigen not present in boiled AS-IR and AS-IS cells.

Antisera produced in coho salmon and in rabbits against *A. salmonicida* antigens (formalin killed cells and extracellular antigens), were tested for their efficacy in protecting coho salmon against experimental furunculosis (Olivier *et al.,* 1985). Results indicated that an avirulent strain was inferior to a virulent strain in its ability to stimulate antibody production for effective passive immunisation of coho salmon. Thus, even though avirulent cells possessed an immunogen that was shown to be well

recognised by rabbits, the immunogen was not relevant to salmon since they received only feeble and inconsistent protection from the antisera.

Passively transferred antiserum which was prepared in rainbow trout to a protease of a virulent strain of *A. salmonicida* provided protection against experimental furunculosis in Atlantic salmon (Shieh, 1985).

Significant protection was provided by a rabbit antiserum to a protease-proficient strain of *A. salmonicida*, particularly against challenge by the heterologous and more virulent protease-deficient strain (Ellis *et al.*, 1988).

In studies to determine the therapeutic and prophylactic efficacy of passive immunisation against *A. salmonicida* infection, rabbit hyperimmune serum was produced against a virulent strain of the bacterium, purified and injected intraperitoneally into brook trout at the time of experimental infection or at various periods after experimental infection. Passive immunisation of brook trout was effective when effected at zero, 24 or 48 h post-infection. Purified immunoglobulins and whole antiserum from rabbits were similarly protective in an experimental bath challenge 35-41 days post-immunisation (Marquis and Lallier, 1989).

Similarly, anti-*A. salmonicida* hyperimmune serum (mammalian immunoglobulin G) was protective for 110 to 150 days post-immunisation (Turgeon and Elazhary, 1992).

Table 1.1.1: Passive immunisation of fish against microbial diseases as conducted by several researchers

Reported by	Disease particular bacterial preparation	Source of immune sera	Fish species passively immunised	Route of Administration	Earliest detection of antibody**	Degree of protection in challenge* Duration of protection	% Mortality Control	% Mortality Immunised
Spence *et al.* (1965)	Furunculosis virulent A.S	Rainbow trout	Coho-salmon	i.p.	ND	4 days	40	10
Cipriano (1981)	Furunculosis virulent A.S	1)Rabbit 2)Salmonid fish	Rainbow trout Atlantic salmon	i.p. "	ND "	-Rabbit Igs did not provide protective immunity +Rainbow trout provided protective immunity		
Cipriano (1982)	Furunculosis virulent A.S	Rainbow trout	Brook trout	i.p.	ND	+protective	70-90	15-20
McCarthy *et al.* (1983)	Furunculosis	Rabbit	Sockeye salmon	i.p.	ND			NG
	1) As-IR virulent strain 2) As-IR " " (boiled) 3) As-IS avirulent					1) +14 days 2) -not protective 3) -not protective		

Table 1.1.1: (continued)

Reported by	Disease particular bacterial preparation	Source of immune sera	Fish species passively immunised	Route of Administration	Earliest detection of antibody**	Degree of protection in challenge* Duration of protection	% Mortality Control	% Mortality Immunised
Olivier *et al.* (1985)	Furunculosis virulent A.S	Rabbit	Coho salmon	i.p.	ND	+protective	90	10
Shieh (1985)	Furunculosis virulent A.S	Rainbow trout antiserum to protease	Atlantic salmon	i.m.	ND	+Protective		NG
Ellis *et al.* (1988d)	Furunculosis	Rabbit	Rainbow trout		ND			
	1)E.C protease proficient strain			i.m. heat inactivated anti-sera		1)Significant protection		NG
	2)E.C protease deficient strain			+ bacteria(F.I)+ i.p. unheated serum(each 0.1 ml)		2)Marginal protection		"
Marquis and Lallier (1989)	Furunculosis virulent A.S	Rabbit	Brook trout	i.p.	ND	35-41days	90	10
Turgeon and Elazhary(1992)	Furunculosis virulent A.S	SHAAS	Speckled trout	i.p.(0.4 ml/kgBW)		110-159 days	90	0
			Atlantic salmon	i.p.	ND	"	87.3	7.3

Table 1.1.1: (continued)

Reported by	Disease particular bacterial preparation	Source of immune sera	Fish species passively immunised	Route of Administration	Earliest detection of antibody**	Degree of protection in challenge* Duration of protection	% Mortality Control	% Mortality Immunised
Harrell *et al.*(1975)	Vibriosis	1)Rainbow trout 2)Rabbit	Rainbow trout	i.p. (0.5ml/30-75g fish)	10 min-1h(peak)	64 days ***	1)100 2)100	5 10
Gunnels *et al.*(1976)	Vibriosis	Rainbow trout	Chinook salmon	i.p.	ND	30 days 52 days	82 93	18 75
Viele *et al.* (1980)	Vibriosis	Rainbow trout 1)Immune plasma 2)Immune leucocyte	Rainbow trout	i.p.	ND	1) 16 days 2) 20-21 days	50 81.3	17.2 31.3
Groberg (1982)	Vibriosis	Coho salmon 1) serum 2) spleen cells	Coho salmon	i.p.	8 h	1)challenge immediately 2)after pass. imm.	46 38	0 20
Salati *et al.* (1989)	Edwardsiellosis	1)Eel 2)Rabbit	Glass eel	Immersion	ND	5 days 5 days(higher degree protection)		
Gutierrez *et al.* (1993)	Edwardsiellosis	Hen's egg yolk Ig Y anti-*E. tarda*	Japanese eel	Oral (Up to 3.8x 10^6cfu bacteria in challenge)	ND	40days	75-100 (prophylactic efficacy)	40-60

Table 1.1.1: (continued)

Reported by	Disease particular bacterial preparation	Source of immune sera	Fish species passively immunised	Route of Administration	Earliest detection of antibody**	Degree of protection in challenge* Duration of protection	% Mortality Control	% Mortality Immunised
Fukuda and Kusuda (1981)	Pseudotuberculosis (*P. piscicida)*	Rabbit	Yellowtail	i.p.	12h(peak)	8 days (highly protective within 24h)	100	30
Olesen (1991)	E.R.M	Rainbow trout	Rainbow trout	i.p.	ND	+protective	91	20
Houghton (1993)	Pancreas disease (viral aetiology)	Affected salmon parr 1)Atlantic salmon (Homogenised kidney material)		i.p.	ND	+significant protection (9 months)		
		2)Experimentally infected fish sera		i.p.	ND	+significant protection		
Hedrick and McDowell (1987)	Channel catfish virus infection	Experimentally infected adult channel catfish	juvenile catfish	i.p.	ND	protective	66-71	1
LaPatra *et al.*.(1994)	IHN	Rainbow trout	Rainbow trout	i.p.	ND		69	6

*Either water-borne or i.p. challenge ** Antibody detection by agglutination test i.p.= Intraperitoneal injection of hyperimmune serum

***protected, but how long not exactly stated C=Control i.m.=intramuscular injection E.R.M=Enteric red mouth A.S= *A. salmonicids*

ND=Not done +=Resistant at challenge but its duration was not investigated -=Not resistant at challenge E.C=Extracellular products NG=Not given
p.i.= Post-immunisation F.I=Formalin inactivated cells SHAAS= An anti-*A. salmonicida* IHN= Infectious haematopoietic necrosis

With respect to passive immunisation against vibriosis, Harrell *et al.* (1975) used anti-*Vibrio anguillarum* serum from rainbow trout (*Oncorhynchus mykiss*) which provided protection against experimental vibriosis in rainbow trout for more than 2 months. Passively transferred anti-*V. anguillarum* rabbit serum also protected rainbow trout. Harrell *et al.* (1975) noted the appearance of serum antibody (measured by agglutination) as early as 10 min after i.p. injection.

Similarly anti-*V. anguillarum* antiserum produced in rainbow trout protected chinook salmon significantly at 30 days post-immunisation. However, the protection was only marginal at 52 days post-immunisation (Gunnels *et al.*, 1976).

Viele *et al.* (1980) immunised rainbow trout by the immersion method against *V. anguillarum*, and used these fish as donors of plasma and cells for transfer to unimmunised trout. Immunity was successfully conferred by plasma taken from donors 16 days post-immunisation (p.i.), and by pronephric and spleen cells from donors 20 and 21 days post-immunisation. Plasma was most effective in transferring protection followed by pronephric cells. Splenocytes gave variable results, while thymic cells were not protective

Whole serum and splenic cells from coho salmon actively immunised against *V. anguillarum* bacterin were injected into non-immunised juvenile coho salmon which were subsequently challenged. The results showed that only serum from parenterally immunised fish (agglutination titre 1:1024) provided complete protection (0% mortality) and splenic cells from the same fish conferred a moderate level of immunity (20% mortality). The mortality rate in control fish when injected with non-immune serum and spleen cells, was 46% and 38% respectively (Groberg, 1982).

The possibility of using passive immunisation with antisera prepared in rabbits as a preventative measure and a medicinal treatment for pseudotuberculosis in cultured yellowtail (*Seriola quinqueradiata*) was investigated by Fukuda and Kusuda (1981). It was suggested that passive immunisation of yellowtail against this disease using rabbit antisera was highly effective for at least 24 h after immunisation.

Salati *et al.* (1989) immunised glass eel against *Edwardsiella tarda* by immersion in aqueous solutions of either eel or rabbit hyperimmune sera to *E. tarda*. They concluded that the procedure provided a degree of protection which increased up to the 5th day post-immunisation. Higher degrees of protection were obtained using rabbit hyperimmune serum. Anti-*E. tarda* IgY from hens vaccinated by injection with formalin-killed cells reduced mortality due to edwardsiellosis in Japanese eel (*Anguilla japonica*) when it was administered orally (Gutierrez *et al.*, 1993). Also, rainbow trout serum from survivors of a challenge with *Renibacterium salmoninarum* (LD_{43}) was used to passively immunise a group of stock fish. On challenge, these fish showed significantly lower mortality rates than non-immunised fish (Campbell *et al.*, 1994).

It was shown that rainbow trout fry injected with serum from previously vaccinated and infected rainbow trout were partially protected against enteric

redmouth (*Yersinia ruckeri*) infection, since only 20% died from the disease compared with 91% of the fry injected with normal trout serum (Olesen, 1991).

Concerning viral diseases of fish, it was shown that injection of homogenised kidney material taken from Atlantic salmon with pancrease disease (possible viral etiology) into Atlantic salmon parr and post-smolts induced very strong protection which was maintained for at least 9 months. No evidence was obtained for a carrier state being established in immune fish. Passive immunisation of salmon parr and post-smolts using convalescent sera from fish experimentally infected with pancreas disease conferred significant protection, suggesting that protection was mediated by antibodies (Houghton, 1993). Nonetheless, it is probable that passive immunisation of this kind may cause the distribution of a viral disease unless the serum is appropriately treated.

Juvenile channel catfish (*Ictalurus punctatus*) were found to be protected against experimental infections with channel catfish virus (CCV) after passive transfer of adult sera containing anti-CCV neutralising activity. This study demonstrated that serum with anti-CCV neutralising activity (presumably due to anti-CCV antibody) obtained from adult channel catfish can protect juveniles from lethal challenge with CCV (Hedrick and McDowell, 1987).

In a recent study, LaPatra *et al.* (1994) injected rainbow trout (mean weight, 4.3 g) with 100 µl of anti-infectious haematopoietic necrosis virus (IHNV) rainbow trout immune serum (IHNV neutralising titre 640) immediately following water-borne exposure to 10^5 plaque-forming units ml^{-1} of IHNV, relative protection was 91% compared to fish injected with normal serum (titre< 20). Fish injected with immune serum at 24 and 48 h post-exposure to virus exhibited relative protection of 88 and 75% respectively. It is suggested from this study that this immunotherapy may have the immediate benefit of reducing mortality.

The possibility of transfer of maternal immunity in young plaice (*Pleuronectes platessa*) (Bly *et al.*, 1986b) was investigated . The study showed that high molecular weight agglutinating antibodies can be induced in the serum and eggs of plaice. Since it is unlikely that plaice oocytes are capable of synthesising antibodies in response to an antigenic stimulus, it can be assumed that antibodies detected in the egg yolk were maternal in origin. Also, maternal immunity in eggs of tilapias (Mor, 1988), in embryo of tilapias (Mor, 1990) and white spotted char (*Salvelinus leucomaenis*) (Kawahara *et al.*, 1993) was investigated. In a recent study, vertical transfer of passive immunity via eggs was examined (Brown *et al.*, 1994). Antibodies produced against *V. anguillarum* in rabbits and in chinook salmon were injected into female coho salmon 11-26 days before spawning. Rabbit antibodies were then detected in eggs. It is suggested from this study that passive vertical transfer of immunity occurs within salmonids but protection is not maintained long after the yolk sac is absorbed.

It is obvious from the previous studies that levels of immunoglobulins (Ig) has rarely been measured after transfer, and the responses of fish to the foreign proteins

(Ig and other proteins) have never been measured. In this study, rate of clearance of antisera transferred to fish, fish response to the antisera and protection conferred by passive immunisation of fish were investigated.

1.1.2 **Active Immunisation:**

Active immunisation is based upon an immune response (both antibody and cell mediated immunity) of an animal to foreign molecules (antigens) e.g. preparations of whole cells or cell extracts. It has been used to combat bacterial, viral and parasitic diseases in fish but requires that there is enough time prior to the probable occurrence of the disease for Ig production (about one month). Active immunisation has worked successfully in particular against vibriosis and enteric redmouth disease.

Active immunisation against vibriosis, streptococcosis and paramoebiasis is discussed at the beginning of each relevant chapter in this thesis.

1.1.3 **Routes of immunisation** :

Vaccines may be administered by different routes. They have been applied to fish by injection, anal and oral intubation (impractical for large number of fish), on food (oral administration), by bathing/ immersion spray or shower. Although there are at least 20 recognised bacterial diseases of salmonids, there are only a limited number of commercial vaccines for the control of the diseases e.g. vibriosis, cold water vibriosis, enteric redmouth, edwardsiellosis and furunculosis. These vaccines, which are mainly whole cell bacterins, are composed of Gram-negative organisms delivered by immersion and intraperitoneal injections (Wong *et al.*, 1992).

Injection:

Intraperitoneal injection: It is usual that fish are anaesthetised not only to facilitate handling but also to avoid excessive stress. The vaccine is usually injected using a multi-dose syringe. Fish are presented ventrally to the needle with the head facing away from the operator so that the needle slides easily beneath the scales. The point of insertion is 2 cm anterior to the pelvic fins, and fairly shallow (<1 cm) with the syringe held at an angle of about 45° to the body surface in order to have minimum risk of damaging the underlying organs. It is also possible to choose an insertion point between the anus and the pelvic fins (Akhlaghi *et al.*, 1993). Injection is not always practical on a large scale and is restricted by the size of fish being immunised.

Most of the time, i.p. injection gives the best results compared to other routes with respect to level and duration of protection (Horne and Ellis, 1988). However, for practical reasons this route has its limitations. Unfortunately, the technique is thought to be slow, and seemed feasible only for large and valuable fish. Intraperitoneal injection is used currently in Norway for commercial vaccines.

Intramuscular (i.m.) injection: In using this route, dorsal muscles may be injected. Leakage of the inoculum before adsorption and muscle necrosis following use of adjuvanted vaccines restrict i.m. injection.

Oral administration:

In this method the antigen is added to the food over a suitable time course. Oral delivery of vaccines has been described as the most desirable method for vaccinating fish (Amend and Johnson, 1981). For extensive aquaculture where fish are farmed in very large ponds and handled only when harvested, oral vaccination is compellingly simple and attractive to many fish farmers. However, the level of protection by oral vaccination has been inferior to other vaccination methods (Gunnels *et al.*, 1976; Gould *et al* ., 1978; Amend and Johnson, 1981; Dec *et al.*, 1990).

In immunising fish by oral route many antigen particles are probably digested by the stomach and gastro-intestinal tract enzymes, especially in carnivorous fish. To evaluate this problem, Johnson and Amend (1983) incorporated a vaccine into gelatin and applied it both orally and anally. They found reasonable protection 59 days post-vaccination by a water-borne challenge with *V. anguillarum* which resulted in 55% mortalities in the control, 0% mortality in the anally vaccinated and 46% mortalities in the orally vaccinated fish. Oral vaccination was the first mass vaccination method used to protect fish against vibriosis (Wong *et al.*, 1992). Effectiveness of an oral enteric coated *Vibrio* 21 (vaccine for use in salmonid fish) (Wong *et al.*, 1992) and uptake of *V. anguillarum* bacterin in the posterior intestine of rainbow trout, sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) after oral administration (Vigneulle and Baudin-Laurencin, 1991) have been reported. Micro-encapsulation techniques may also be important for bypassing the deleterious effects of the upper region of gastro-intestinal tract and development of successful oral vaccines.

Immersion method:

Bath vaccination: The ease of addition of vaccine directly to the tank or pond without netting or handling has led to the increasing use of the bath method (Horne and Ellis, 1988). The water level of the holding tank is lowered to the minimum level tolerated by the fish. Vaccine is added to this volume to achieve a dilution usually of (depending on the brand of vaccine) one part in 500 and fish are left for a specific amount of time. Bath vaccination is easy to carry out since it is not labour intensive. Antigen is in direct contact with the body surfaces and gills and thus available to be translocated into the body. Many researcher have reported the benefits of immersion vaccination (Egidius and Andersen, 1979; Håstein *et al.*, 1980; Song *et al.*, 1980; Amend and Johnson, 1981). This method is preferred with salmon as the fish need not to be netted

Immersion vaccination for 30 s at 1/10 dilution is usually called dip vaccination. This appears to be the most popular method for trout. With the use of a small tank

containing vaccine in close proximity to the holding tanks, netted fish are immersed in the diluted vaccine for 20 s to 1 min according to the vaccine manufacturer's recommendation. According to several workers, immersion of fish in hyperosmotic 5-8.5% NaCl before immersing in the vaccine has provided a high level of protection in comparison with simply dipping fish into vaccine. Apparently the vaccine solution substitutes for fluids which have been lost in the hyperosmotic solution. As a result, more bacteria cross the fish's body external barriers.

Formerly, considerable attention was focused on hyperosmotic infiltration (Croy and Amend, 1977; Aoki and Kitao, 1978; Nakajima and Chikahata, 1979; Antipa *et al.*, 1980). It is now appreciated that the technique is extremely stressful to fish (Busch *et al.*, 1978), and that the level of protection is not greater than the much simpler direct immersion method (Antipa *et al.*, 1980).

Treatment of fish with formalin and pH from 6-8 did not affect protective immunity and fish can be equally immunised in fresh water or salt water (Amend and Johnson, 1981). Field experience has also indicated that different levels of water hardness does not influence effective immunity (Amend and Johnson, 1981) but other chemicals need evaluation to verify that they do not interfere with uptake of the antigen by the fish.

Automated vaccination:

This was attempted by Gould *et al.* (1978) using a spray applicator which has since been extensively used on large farm units in the USA. In this method of vaccination, fish are drawn onto a conveyor belt and passed beneath a spray set or dipping process to ensure at least a 5 s exposure to the diluted vaccine.

1.2 **Fish defence mechanisms**:

The fish immune response to many diseases is well defined. The resistance is specific to the challenging pathogen and persists for a relatively long period of time (Ellis, 1988a).

Thymus , kidney and spleen (Ellis, 1988b) and gut (Rombout *et al.*, 1993) are the major lymphoid organs of teleost fish . During the first few weeks post-hatching the rate of growth of these tissues in rainbow trout is faster than the rest of the body and the weight of the lymphoid organs, relative to body weight, reaches a peak at 2 months of age, when fish are 0.5 g (Tatner and Manning, 1983a) and after that their relative weights decrease with age. The immune responses of fish can be divided into non-specific and specific components:

1.2.1 **Non-specific defence mechanisms:**

Integumentary defence mechanism:

The integumentary system of the fish forms the primary defence barrier between the fish and its environment. The fish body is covered by a slippery layer of protective mucus which is physically shielding against osmotic imbalance and against pathogens. The mucus layer provides important mechanical protective functions preventing bacteria from gaining intimate contact with the living epidermal cells (Austin and Austin, 1993). Internally, mucus also coats the wall of the alimentary tract.

The inflammatory response:

The inflammatory response is the other non-specific defence mechanism which provides a site of interaction with the cells and humoral factors best able to destroy or limit the spread of injurious agents such as living organisms or non-living agents such as trauma, radiant energy, chemicals and toxins. The inflammatory responses are vasodilation and increased blood flow and vascular permeability. Exudation of plasma and migration of monocytes, macrophages, neutrophils and cytotoxic cells into the tissues occur to facilitate removal of foreign particulate material.

Macrophages have a vital role in many aspects of the immune response in fish. As well as being accessory cells important in initiating specific immune responses, they are also potent effector cells capable of killing a wide range of pathogens (Secombes, 1990). *In vivo* and *in vitro* studies have demonstrated that macrophages are highly phagocytic for inert and antigenic material (Ellis *et al.*, 1976; McKinney *et al.*, 1977).

Elevated levels of antibodies specific to antigens can enhance phagocytosis in fish (Griffin, 1983; Sakai, 1984; Scott *et al.*, 1985; Honda *et al.*, 1986). In rainbow trout immunised with *V. anguillarum*, phagocytic activity of macrophages increased significantly 5 weeks after immunisation as compared to the activity of macrophages from normal fish (Honda *et al.*, 1985).

Neutrophils have been reported to have no phagocytic properties (Ellis *et al.*, 1976; McKinney *et al.*, 1977) or to be only weakly phagocytic (Young and Chapman, 1978). Ellis (1981) has suggested that the fish neutrophil may carry out a bactericidal role extracellulary rather than intracellulary.

Regarding the protective role of pigment-containing macrophages and melanocytes which are found in the lymphoid tissues of most teleosts, it is postulated that the melanin contents of these cells in fish viscera may provide a means of protection from free-radical damage, similar to its function in protection from radiation-induced free-radicals in the skin (Ellis, 1981).

The complement system:

The complement system of higher vertebrates plays a prominent role in humoral and cellular immunity against pathogens and the inflammatory process which results in the killing and elimination of cellular antigens (Ingram, 1990a). Harrell *et al.* (1976) demonstrated that heat-stable (presumably antibody) and heat-labile (presumably complement) components were necessary to prevent the growth of *V. anguillarum* in an *in vitro* experiment with trout immune serum and mucus. The complement functions either as an enzyme or binding protein in 1) classical (or antibody-dependent) pathway, 2) alternative (or antibody-independent) pathway (Ingram, 1990a). For example, the sera of brown trout (Ingram, 1987), albacore tuna (Fujii *et al.*, 1979; Giclas *et al.*, 1981), rainbow trout (Sakai, 1983a) and several other teleost species, contain complement components capable of fixing Ig via the classical pathway. Carp (Kaastrup and Koch, 1983), and rainbow trout sera (Nonaka *et al.*, 1981; Sakai, 1981b, 1983b) also display spontaneous antibody-independent haemolysis indicative of an alternative pathway (AP) (Ingram, 1990a). Jenkins and Ourth (1993) also determined that the alternative complement pathway functions in opsonophagocytosis in catfish and the ingestion by neutrophils was especially enhanced. Because complement is constitutively present *in vivo*, its opsonic function via the AP may be of greater importance than opsonins such as C-reactive protein (Kodama *et al.*, 1989).

The complement activity of fish serum has been pointed out as an important innate defence against bacterial pathogens (Trust *et al.*, 1981). Nonaka *et al.* (1981) have demonstrated the ability of trout IgM to fix trout complement and activate its lytic properties. It is suggested that for antibody-activation of complement the two components must be derived from fairly closely related species (Sakai, 1981b). However, Ingram (1990a) reported that brown trout antibodies are capable of fixation of guinea pig complement as well as trout serum in a complement fixation test. Purified factor D of carp (*Cyprinus carpio*) complement homologous to mammalian factor D was proven to be an alpha-globulin with a molecular mass of 29 kDa and the serum concentration was estimated to be 6 $\mu g\ ml^{-1}$ (Yano and Nakao, 1994).

Regarding the opsonising effect of anti-*V. anguillarum* rainbow trout serum, Honda *et al.* (1985) showed that the opsonising effects of the serum increased with increasing amount of antibody in cultures. These workers also showed that rainbow trout complement enhanced the opsonising effect of the antibody, phagocytic activity then increased significantly in the presence of antibody. Fish antibody also, in the presence of normal serum as a complement source is capable of lysing target cells (Nonaka *et al.*, 1981).

Opsonisation and the related complement-binding are of major importance in optimising phagocytosis. Phagocytosis by fish leukocytes increased following opsonisation of bacteria with specific antibodies alone (Song and Kou, 1981; Griffin,

1983) or with specific antibodies and complement (Sakai, 1984). However, Olivier *et al.* (1986) concluded that the principal opsonin in fish serum is complement.

Honda *et al.* (1985) demonstrated that the phagocytosis of *V.anguillarum* by normal peritoneal macrophages was enhanced in the presence of antibody and complement. The optimum temperature to inactivate complement of fish sera differs between species, e.g. 44°C for rainbow trout, 45°C for coho salmon and goldfish (*Carassius auratus*) and 47°C for tilapia (*Sarotherodon nilotica*) all for 20 minutes (Sakai, 1981a).

The other non-specific defence factors:

A number of non-specific defence factors have been identified in fish ova including C-reactive protein and lectin-like agglutinins (Ingram, 1980).

Lysozyme, which is a mucolytic enzyme capable of destroying the mucopeptides in bacterial cell walls, exists in rainbow trout at a concentration about 15 times higher than the concentration found in brown trout and Atlantic salmon (Lie *et al.*, 1989). Lysozyme has been purified from plaice (Fletcher and White, 1976), and from rainbow trout (Grinde, 1989). Macrophages may actively secrete various substances such as lysozyme and interferon (which confer viral resistance) (Agius, 1985).

Many factors may influence the function of non-specific defence mechanisms in fish such as age, nutritional status, endocrine function, etc. For example, Durve and Lovell (1982) found that feeding a level of ascorbic acid higher than minimum dietary requirement for normal growth provided increased resistance of channel catfish fingerlings to the pathogenic bacterium *Edwardsiella tarda*, especially at low temperatures (23°C).

Fish depend more heavily on non-specific defence mechanisms than do mammals (Anderson, 1992). This is especially true for short-lived fish living in cool or cold waters because the development of a specific immune response is temperature dependent (Avtalion, 1981).

1.2.2 **Specific defence mechanism:**

Teleost fish may not become immunologically competent until some weeks post-hatching. It seems that in this period the lymphoid system is still developing. While the vaccination of salmonids of 4 g or larger resulted in durable immunity (a year or longer), vaccination of 1 g fish resulted in a much shorter term of protection (Amend and Johnson, 1981; Johnson *et al.*, 1982a, b). In fish, the ability to establish immune memory and to produce antibody to T-dependent antigens develops by about 8 weeks post-hatch (Ellis, 1988b). Also, the ability of salmonid fish to develop prolonged duration of protection (long term memory) after vaccination is achieved when fish are about 4 g (Ellis, 1988b).

The specific immune response occurs in deep-lying lymphoid tissues in the kidney and spleen which collect the antigen and are stimulated to produce antibodies that are released into the circulation (Chiller *et al.*, 1969).

The kidney is the main antibody-producing organ. It contains a generalised haemopoietic tissue rich in lymphocytes and plasma cells as well as many macrophages which phagocytose antigens (Ellis, 1988b). The spleen contains fewer haemopoietic and lymphoid cells than the kidney, being composed mainly of blood held in sinuses. It, however, contains specialised capillary walls, termed ellipsoids, which are composed of reticulin fibres and macrophages (Ellis, 1988b). The reticulin fibre network is specialised for trapping immune-complexes while the macrophages are highly phagocytic (Ellis, 1980).

Immunoglobulins (Ig) are found in most of tissue fluids in fish (plasma, lymph, skin and gut mucus and bile) since the blood vascular system of fish is evidently quite permeable to serum Ig (Ellis, 1982).

Ig comprise 40-50% of the total blood serum proteins (Ellis, 1982). The total protein of uninjected brown trout (*Salmo trutta*) was 5.11 ± 0.71 g 100 ml $^{-1}$ (Ingram and Alexander, 1977). Wardle (1971) demonstrated that the protein content of the serum and that of lymph from the neural lymphatic duct of the plaice was qualitatively identical and lymph contained as high as 80% of the protein concentration of the plasma.

Fish Ig exist as only one class that resembles mammalian IgM (Litman, 1975). It is tetrameric in species such as salmonids and carp (Shelton and Smith, 1970; Litman, 1975), composed of 4 subunits each with 2 heavy and 2 light chains (Ellis, 1982). Chondrichthyean fish also possess only IgM but in pentameric and monomeric forms (Ellis, 1982). The half-life of serum antibody in fish is as long as 12.5 days in carp (Avtalion *et al.*, 1976) and 16 days in the sheepshead (*Archosargus probatocephalus*) for both high and low molecular weight Ig (Lobb and Clem, 1981a). In contrast, it was as short as 49 h in coho salmon (Voss *et al.*, 1980).

Anti-bacterial and anti-hapten antibody activity has been ascribed to a tetrameric (~800,000 daltons) immunoglobulin found in sera of coho salmon (Voss *et al.*, 1980). Agglutinating activity is useful towards particulate antigens such as bacteria, and precipitating activity is particularly important in neutralising soluble toxins. Agglutinating antibody is readily demonstrated in fish serum whereas precipitating antibody activity is not (Ellis, 1982). Hodgins *et al.* (1967) found rainbow trout possessed only HMW IgM and could detect passive haemagglutinating antibodies to bovine serum albumin early in the response but precipitating activity was not detected until much later. Ingram and Alexander (1986) investigated immune responses in trout injected with haemocyanin. It was concluded from that work that the formation of precipitins seems to depend upon the nature, size and dose of antigen. High doses of antigen are often required to stimulate precipitin formation (Ingram and Alexander,

1986). However, elevated level of antibodies specific to antigens do not necessarily relate to protection against disease (Munn *et al.*, 1982; Cipriano, 1983).

Active vaccination by i.p. injection results in enhanced levels of antibodies in cutaneous mucus and perhaps in intestinal mucus. Rainbow trout injected with heat-killed *V. anguillarum* cells in adjuvant (Harrell *et al.*, 1976) and injected with *R. salmoninarium* in adjuvant (Paterson *et al.*, 1981), produced elevated levels of antibodies in the skin mucus as well as in serum. Fletcher and White (1973) and Paterson *et al.* (1981) also examined the intestinal mucus for its antibody content; antibodies were detected but the titre were only marginally enhanced by injection of antigen.

With respect to Ig in fish secretions, Fletcher and Grant (1969) reported that the mucus IgM of plaice was similar to serum IgM. Antibody production could be stimulated systemically or locally, depending on the method of antigen administration (Fletcher and White, 1973). In another study Harrell *et al.* (1975) found anti-*V. anguillarum* agglutinins with titres of 1: 240 in the ovarian fluid of rainbow trout, however, none was found in the eggs. Also mucus and bile of the sheepshead contained IgM and although it was not derived by exudation from the serum Ig pool, it was antigenically identical to the serum Ig (Lobb and Clem, 1981a,b).

Regarding maternal antibodies and the reason why fry of some species are relatively resistant to the pathogens, this has been postulated to be related to humoral substances (maternal antibodies) which may play a defensive role. However, immunoglobulin does not appear to be present in the ova of salmonid (Ellis, 1988b).

Apart from the humoral immune response, cell-mediated immune (CMI) responses are often a factor in the protection of fish against disease (Smith *et al.*, 1980; Aoki *et al.*, 1984). CMI detected by using a mixed leucocyte response in Atlantic salmon developed at day 42 post-hatch, (Ellis, 1977) and allograft rejection responses developed in carp and rainbow trout by 16 days (Botham and Manning, 1981) and 14 days (Tatner and Manning, 1983b) post-hatch respectively. Thus, CMI matures a little earlier than the humoral immune response (in 2-4 weeks post-hatch) particularly to T-dependent antigens, e.g. HGG, and functions through the production of cytotoxic T-like cells (Ellis, 1988b).

The delayed-type hypersensitivity (DTH) skin reaction provides a convenient indicator of sensitised T lymphocytes in mammals. Rainbow trout immunised with seal worm extract produced a typical DTH reaction to live sealworm challenge which supports the existence of T-cell function with anamnesis in fish exposed to sealworm antigens. However, when the test fish were immunised with live sealworm and later challenged with live sealworm, the secondary response was poor and showed no difference from control fish (Ramakrishna *et al.*, 1993).

Thomas (1988) observed a swelling response 72 h after sonicated *Cryptobia* antigen was injected subcutaneously at the peduncle of rainbow trout previously

infected with the parasite. Histological examination of the swelling reaction demonstrated the presence of mononuclear cell infiltrates

With respect to acute hypersensitivity responses in fish, it was reported that immediate hypersensitivity skin reactions occurred in several flatfish species induced by C-polysaccharide substances (Baldo and Fletcher, 1975). However, typical mammalian-like mast cells are not present in fish (Ellis, 1982) and the role of histamine has not been definitively demonstrated. In a recent work, mast cells are demonstrated in the swimbladder of Atlantic salmon (Reite and Evensen, 1994).

With respect to the immune tolerance, rainbow trout fry gave good protective immune responses to *V. anguillarum* when vaccinated by direct immersion and i.p injection, from a weight of 0.4 g onwards. Prior exposure to the antigen did not lead to tolerance induction. Possibly before that time (0.4 g), the immune system is capable of becoming tolerant and the external exclusion of antigens in the water is a protective mechanism by the fry to protect its immature immune system. Indeed a period of unresponsiveness to the vaccine antigen occurs before fish reach the critical weight (Tatner and Horne, 1984). The phenomenon of oral tolerance in rainbow trout has been investigated by Davidson *et al.* (1994). It was found that pre-exposure to antigen daily via the oral route for 5-10 days or by a single i.p injection was found to have no effect on the systemic response to human gamma globulin (HGG) administered i.p. 35 days later. When the same oral route experiment was performed using *A. salmonicida* as an antigen no suppression of the systemic antibody response was observed, indeed, an enhancement of the response was apparent.

Immersion vaccination provides protective immunity against vibriosis up to one year (Alexander, 1990). In contrast, the activation of most of the non-specific defence mechanisms may not last as long as the specific immune response (Finn and Nilsen, 1971; Gosting *et al.*, 1981)

1.3 **Immunostimulants and role of adjuvants in fish vaccines:**

Immunostimulants or adjuvants are substances which enhance the non-specific defence mechanisms or the specific immune response generating elevation of circulating antibody titres. They may be used alone inducing elevated activities in the non-specific defence mechanisms such as increased oxidative activity of neutrophils, augmented engulfment activity of phagocytic cells or potentiation of cytotoxic cells so as to increase the resistance of fish to various infectious diseases (Anderson, 1992).

Different adjuvants have been investigated by Duier (1985) in fish. The results of this study indicated that the use of a suitable adjuvant can maintain the antibody titre at a high level for many months, protecting trout throughout nearly all of its economic life with no need of a booster injection.

In cases where disease outbreaks are cyclical or predictable, or prior to stressful events, losses can be reduced by use of immunostimulants to enhance non-specific defence mechanisms (Anderson, 1992). Zeeman (1986) found the stress of smolting

depressed the immune response in coho salmon and suggested immunostimulants could be used to restore normality at this time.

Oil based adjuvants, such as Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA), act as depots or reservoirs for holding the antigens in tissues after injection. The *Mycobacterium* sp. component of FCA is a T-cell stimulator. In fish the immunomodulatory effects of these adjuvants on the humoral immune response, especially to T-dependent antigens, can be considerably enhanced by administering the antigen with adjuvants (Ellis, 1988c). Simple mineral oil-based adjuvants (FCA and FIA) resulted in protection against furunculosis, vibriosis and enteric red mouth disease. The addition of whole cell bacterin to the adjuvant doubled protection (relative per cent survival= RPS) to about RPS= 40% and increasing the administration volume also improved protection (Adams *et al.*, 1988). Fish as small as 1.2 g produced antibodies as early as 4 weeks after i.p. administration of *A. salmonicida* cells in Freund's complete adjuvant. High levels of antibody persisted for at least 1 year after injection and provided juvenile coho salmon with protective immunity against furunculosis (Paterson and Fryer, 1974).

Bacille Calmette Guerin (BCG), an attenuated bovine strain of *Mycobacterium tuberculosis* in combination with a *Y. ruckeri* bacterin has been injected into rainbow trout. Bacterial clearance was increased, however, enhancement of other immunological parameters, such as agglutination and serum bactericidal activity, was inconclusive (Grayson *et al.*, 1987). It was concluded that while BCG is a well recognised immunostimulant in mammals, the colder environment of the fish may slow its action (Anderson, 1992).

From the experiments conducted by Horne *et al.* (1982) it was shown that vaccination of fish annually with vaccine containing 2.5% w v^{-1} potassium aluminium sulphate adjuvant early in the season, before the occurrence of temperatures at which vibriosis outbreaks become frequent, was a worthwhile procedure.

Quil-A and other detergents and quaternary ammonium compounds may affect the cell membrane or epidermis, and loosen intracellular junctions. Therefore, the absorption of antigen can be facilitated (Maharaj *et al.*, 1986). The potential of enhancing enteric uptake of orally and anally delivered antigen (human gamma globulin; HGG) with Quil-A saponin adjuvants in tilapia (*Oreochromis mossambicus*) was investigated by Jenkins *et al.* (1991). They successfully enclosed the HGG protein in the form of micelles or immune stimulatory complexes (ISCOMS) and administered it to the fish orally and anally. Maximal concentrations of the antigen occurred in intestinal tissues six hours later when given orally; whereas, the antigen given by the anal route reached maximal levels in 1 hour after administration.

The effect of oral administration of different immunostimulants on the defence mechanisms of fish have still to be confirmed. However, immunomodulatory effects of immunoactive peptides from the fermented products of chicken egg (EF 203) administered orally to rainbow trout resulted in increased chemiluminescent responses

of kidney phagocytes and increased resistance to both natural and experimental beta-haemolytic streptococcal infection (Yoshida *et al.*, 1993).

Levamisole is known to be a T-cell stimulator and immunomodulator. It can act alone to elevate the non-specific defence mechanisms in fish, or it can be used as an adjuvant with a vaccine. Levamisole protected rainbow trout against *V. anguillarum* at a dose of 5 mg kg^{-1} (Kajita, 1990). The induced protection seemed to be due to a combination of serum bactericidal activity, complement activation, phagocytic elevations, and natural killer cell enhancement .

Adams *et al.* (1988) evaluated the potency of bath vaccine in Atlantic salmon. Booster vaccinations using *A. salmonicida* whole cells were effective against furunculosis (RPS= 48.5%), when given 4 weeks after the primary vaccination. A single vaccination using whole cells extracted with the non-ionic detergent Triton-X-100 resulted in protection against furunculosis (RPS= 29.9%) while bacteria treated with ethylenediamine tetra acetic acid (EDTA) gave little or no protection. Untreated whole cells gave RPS of 9.9%. Extracellular products (ECP) from *A. salmonicida* were effective in protection when presented in particulate form, by binding ECP to polystyrene beads (RPS= 35.6%). Soluble ECP vaccine provided no protection at all.

Lipopolysaccharide (LPS) extracted from gram-negative bacteria such as *Salmonella typhimurium* are used as an immunostimulant. LPS stimulates B cell proliferation *in vivo* or *in vitro* in appropriate doses (Anderson, 1992).

Phagocytic activity of leucocytes of fish treated with chitin as an immunostimulant was investigated by Sakai *et al.* (1991a). No increase was observed in the lysozyme and haemolytic activity of fish injected with chitin. The LD_{50} of *V. anguillarum* in chitin-injected fish was about 10 times higher than in control fish.

Glucan, a long-chained polysaccharide cell wall extract from yeast, is reported to be a stimulator of non-specific defence mechanisms in animals, including fish (Yano *et al.*, 1989; Robertsen *et al.*, 1990).

Chen and Ainsworth (1992) reported that ß-1, 3 glucan administration enhances the phagocytic activity and bactericidal ability of anterior kidney phagocytes in channel catfish challenged with *Edwardsiella ictaluri*. Jørgensen *et al.* (1993) found that yeast glucan injected intraperitoneally in Atlantic salmon increased the ability of anterior kidney macrophages to kill a virulent strain of *A. salmonicida*. The macrophage response of injected glucan was evident 2 days post-injection. They also found that the increased phagocytic activity of macrophages was the result of increased activity rather than increased numbers.

In a recent study, Ramadan *et al.* (1994) studied the effects of feeding ascogen on the immune response of tilapia to *Aeromonas hydrophila* vaccine. The results showed that ascogen had a marked immunopotentiating effect (both humoral and cell-mediated) after i.p. injection as well as after direct immersion.

Immunostimulants, adjuvants, and vaccine carriers and their functions, used routinely and experimentally in fish and other animals are summarised in appendix VIII.

1.4 Uptake of Antigen and other substances:

Antigen uptake may occur through the skin, lateral line, the gill, or even by the fish drinking small amounts of antigen (ingestion). Fish skin including mucus, generally forms a three layered barrier between the host and its external environment. Fin tissues of fish, because of their membranous form, provide concomitant accessibility to blood. Fish nares are blind pits where water is channelled over sensory nerves. Figure 1.1 shows the possible sites of uptake of antigens.

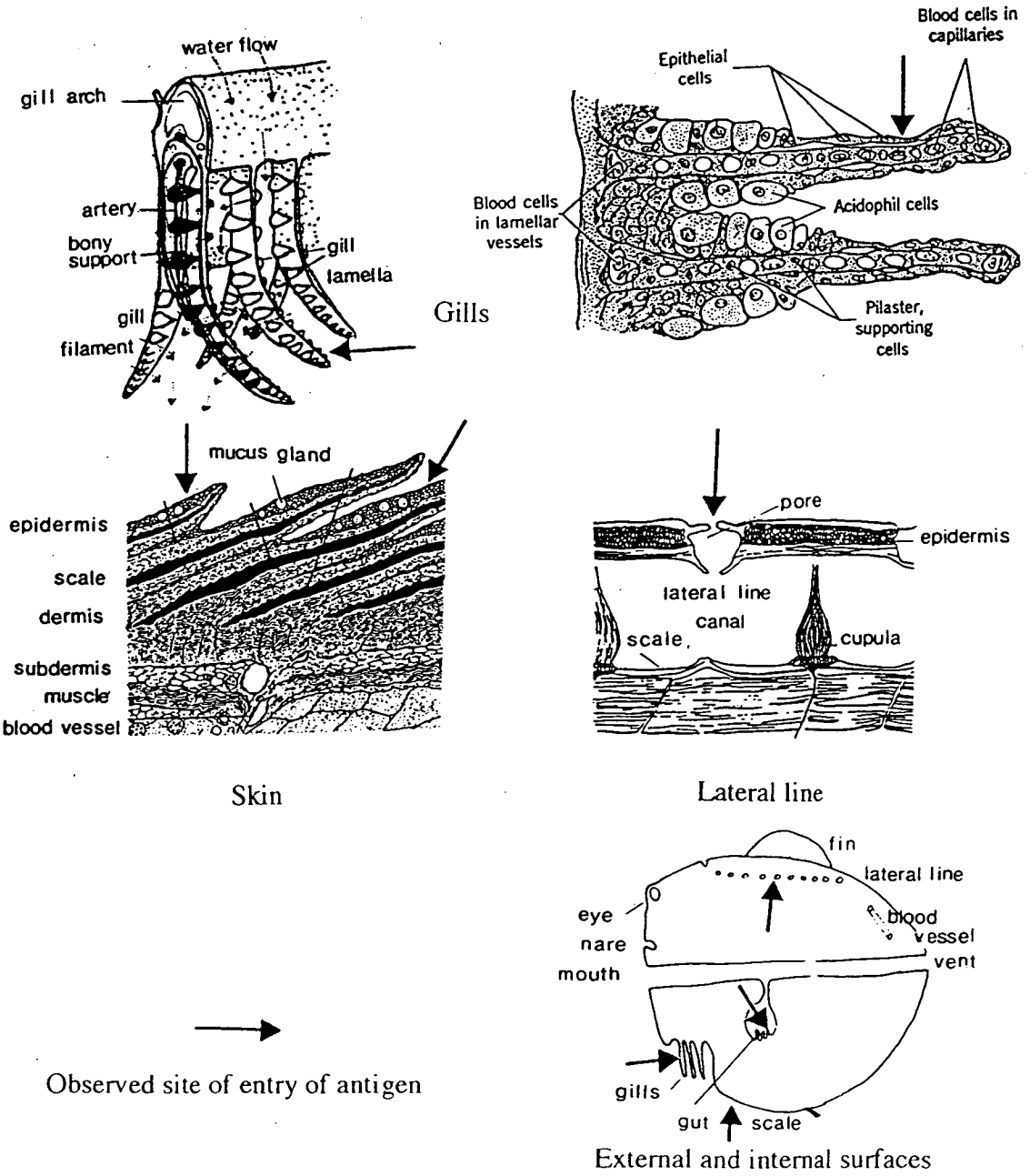


Figure 1.1: Possible sites of fish body surfaces for the uptake of antigen

Amend and Johnson (1981) observed that protective *V. anguillarum* antigen (LPS) can induce a specific immune response in fish by short simple immersion or shower. Antigen is somehow taken up across the external surface (body surface) and results in stimulation of lymphocytes which release the antibody into the serum. However, it is not known precisely how the antigen attaches to the surface of the fish and how it is transported to the immune sites within the fish.

Moore *et al.* (1994) exposed 1-5 g rainbow trout with a continuous or 'flush" exposure to 10^7 particles ml^{-1} for 24 h in a solution of fluorescent latex beads covalently conjugated with bovine serum albumin. Beads were observed primarily within cells in the epithelium of gill filaments and scaled epidermis, where the greatest uptake occurred adjacent to the lateral line.

Using both light and electron microscopy Zapata *et al.* (1987) examined the uptake of antigen by salmonid gill cells after bath immunisation with a *Y. ruckeri* O-antigen bacterin at different doses. The particulate antigen was observed in the gill mucus, adhering to and within the pavement cells covering the gill filament and in mononuclear phagocytes below the epidermal gill cells. They also found a direct dose-response correlation in the observed immune response according to the number of splenic antibody-producing cells 14 days after immunisation.

Other evidence for permeation of gills by bacteria is work by Tatner and Horne (1983) following hyperosmotic infiltration of bacterial immunogen. Filaments and secondary lamellae of gill tissues increase the surface-area-to-volume ratio for gas exchange and offer a readily accessible supply of blood to a diversity of pathogens.

The lateral line, is a sensory canal system underlying the epidermis of some fish and has numerous pores opening to the epidermis. Amend and Fender (1976) postulated that the perfusion of immunogen in hyperosmotic infiltration was via the lateral line system, then by cross-diffusion into neighbouring lymph ducts and so into the venous system.

Juvenile rainbow trout were immersed (10-60 s) in a bath containing 2.5 mg horse- radish- peroxidase (HRP) in order to determine the tissue localisation of HRP. was detectable within all tissues examined as early as 5 min post treatment regardless of immersion period. Peak net tissue accumulation of HRP on a per g wet weight basis was found to be of the order; spleen > kidney >heart> liver. Peak tissue level of HRP was recorded following 60 s exposure to the antigen (McLean *et al.*, 1989).

Many factors seem to affect the efficacy of the uptake of a ^{14}C-labelled *V. anguillarum* cells by direct immersion of rainbow trout including duration of immersion and the concentration and temperature of the vaccine bath (Tatner and Horne, 1983).

Several studies have been conducted on uptake of vegetable enzymatic protein and HRP in the gut of fish without digestion (Watanabe, 1982). Observations by light and electron microscopy of the uptake of rabbit immunoglobulin against HRP by the columnar epithelial cells of the gut of rainbow trout suggested that the columnar

epithelial cells took up the protein by pinocytosis through the pits between microvilli without enzymic digestion and the most active uptake was found in the posterior gut (Fujino *et al.*, 1987).

However, antigen administered orally is partially inactivated by the digestive enzymes in the upper alimentary canal prior being able to reach the intestine where antigen uptake can take place. This is shown from results of studies for common carp (McLean and Ash, 1986) and for rainbow trout (McLean and Ash, 1987) which revealed that in both cases, the tissue presence of orally administered HRP is significantly (1000-fold) greater in the "agastric" carp than in the "gastric" trout.

Therefore, a method of protecting the antigen from the digestive enzyme in the alimentary canal is required, for instance, conjugation with carriers and micro-encapsulation of an antigen by coating with cellulose derivatives can be investigated. McLean and Ash (1990) examined the protective effects of the antiprotease, soy-bean trypsin inhibitor (SBTI) and a synthetic detergent which enhanced both tissue and plasma levels of HRP, enhanced accumulation of HRP was noted when it was delivered in SBTI- HRP-detergent form.

Jenkins *et al.* (1991), investigated the potential of enhancing enteric uptake of orally and anally delivered antigen (human gamma globulin) with Quil-A saponin adjuvants in tilapia. They used several sensitive immunohistochemical and immuno-electronmicroscopical techniques. The results demonstrate the ability of the intestine of tilapia to absorb and to have potentially relevant immune interactions with protein antigens delivered by these routes and the potential of enhancing antigen absorption with saponin adjuvants.

Several factors influence antigen uptake, including pH and salt concentration of the vaccine solution, vaccine concentration, length of vaccination time, water temperature during vaccination, stress, size of fish, anaesthetics, and the physio-chemical characteristics of the antigens, such e.g. (particulate or soluble) (Tatner, 1978; Tatner and Horne, 1983).

1.5 **Effects of temperature on the immune responses of fish**:

It is well documented that as fish are poikilothermic animals, temperature has a profound effect on the length of the induction period of immunity. Generally, the colder the water temperature, the longer the time for the development of protective immunity. It takes 14 to 21 days in salmonids for the development of protective immunity after administration of *V. anguillarum* vaccine at 15°C. If the temperature is lowered to 5°C this time will be extended to at least 40 days. At very low temperatures, for example 4°C and less, the immune system will not react at all, while higher temperatures will shorten the induction period (Smith, 1988). However, Lillehaug *et al.* (1993) showed that groups of Atlantic salmon parr vaccinated either by injection or immersion against cold water vibriosis caused by *V. salmonicida* and kept initially at 2, 4, 6°C for five weeks and then 10°C, showed better protection against infection than fish vaccinated at higher temperatures (8 or 10°C). The mean

levels of antibodies against *V. salmonicida* were not higher in the low temperature group, indicating that mechanisms other than antibody also play a role in protective immunity.

Temperatures which are low but still within the normal physiological range of a particular species of fish (e.g. less than 6°C for rainbow trout or 12°C for carp) can depress the adaptive immune response in a variety of ways (Avtalion, 1981). However, phagocytosis seemed less affected by temperature change (Avtalion, 1981).

The first appearance of humoral antibodies in coho salmon injected with killed *V. anguillarum* cells occurred after about 25 days at 6°C but after only about 10 days at 18°C (Groberg, 1982). This phenomenon must be taken into consideration when planning vaccination so that the fish are immune by the time they are exposed to the pathogen.

In an attempt to understand the interrelation between environmental temperature and immune competence, Bly and Clem (1991) found that *in vivo* exposure to an "ultra-low temperature" (when temperature can drop to as low as 2°C and fluctuate as much as 9°C during a 12h period) can have profound suppressive effects on immune function in channel catfish. Furthermore, this form of suppression was not a typical stress-induced response, i.e blood serum chemistry and blood cell counts did not change in a manner consistent with transport induced stress.

Yamaguchi *et al.* (1981) showed that the rainbow trout immune system could be affected by seasonal changes in temperature. According to their results it seems that the immune system of the fish responds more effectively in the spring than in the autumn under apparently uniform conditions of light. When the environmental temperature is constant, antibody synthesis in fish is still affected by the season.

The occurrence of circulating antibodies against *V. anguillarum* 01 antigen was highly variable in vaccinated rainbow trout and was closely related to season but not geographic location. Percentages of seropositive fish generally ranged from 0-40%. Circulating antibodies tended to be undetectable in fish sampled in spring. Maximum percentages of seropositive fish occurred in summer and autumn (Williams and Lindqvist, 1992).

1.6 **Immunological assays**:

Many assays have been employed for detection of the fish antibody response. Bacterial agglutination (Roberson, 1990), fluorescent antibody test (Anderson, 1990a), passive hemolytic plaque assay for detecting antibody-producing cells (Anderson, 1990b), complement-fixation test (Ingram, 1990a), immunoelectrophoresis (Ingram, 1990b), and counter-current electrophoresis (Ingram, 1990c) have been employed in fish immunological studies.

ELISA (enzyme-linked immunosorbent assay):

The enzyme linked immunosorbent assay is currently receiving the most attention due to its sensitivity, specificity , speed, versatility, low cost, and reagent stability. In the indirect ELISA, antigens are adsorbed to the solid substrate and the sera (antibody) to be measured is added. The secondary antibody (anti-antibody that is usually loosely referred to as the conjugate) which is already conjugated to an appropriate enzyme is then added and if any antibody in the sera has been bound, it will attach to it.

The sensitivity of ELISA was several times higher than that of agglutination test to detect anti-*A. salmonicida* antibody (Kodama *et al.*, 1985a). The result of agglutination and ELISA tests for detection of anti-*V. anguillarum* antibodies in salmonids after different routes of vaccination showed that the agglutination test was less accurate than the ELISA (Munday *et al.*, 1992). ELISA proved to be more sensitive than the agglutination test for detection of antibodies to *Y. ruckeri* in rainbow trout (Olesen, 1991).

ELISA fulfills all the requirements of a rapid, sensitive, simple, and highly reproducible assay. The reagents are also inexpensive and do not have a limited shelf-life or the health hazards associated with utilising radio-isotopes (McArthur and Sengupta, 1982).

Over the last two decades, ELISA has been used a great deal for immunological surveys and with a variety of procedures. Following are examples of different ELISAs that have been used.

Roberson (1981) used thin-layer ELISA for the detection of fish antibody against *V. anguillarum* and *Y. ruckeri* O-antigen. The sensitivity of the enzyme-linked reaction was found comparable to passive hemagglutination.

Also, two indirect ELISAs were developed to evaluate specific antibodies against *Y. ruckeri* and Egtved virus in rainbow trout. The sensitivity and specificity of each ELISA were compared with agglutination and seroneutralisation (Cossarini-Dunier, 1985).

Hamilton *et al.* (1986) successfully monitored antibody production during experimental *A. salmonicida* infection in rainbow trout with a modified ELISA.

Using ELISA, Hongslo *et al.* (1987) showed that both vaccination by i.p. injection and vaccination by immersion induced a high degree of protective immunity against vibriosis which lasted for at least 46 weeks. At each experimental infection the proportions of seropositive fish were lower than the proportions of fish with protective immunity. However, seropositive individuals were found in both experimental groups throughout that study.

Intraperitoneal vaccination of rainbow trout with *V. anguillarum* at varying temperatures and seasons was conducted by Thorburn *et al.* (1989), who classified fish as sero-negative (low titre) or sero-positive (high titre). They tested for anti-*V. anguillarum* serotype I antibodies with ELISA. A high percentage ($\geq$ 75%) of trout in

all i.p-vaccinated groups were sero-positive during most of the growing seasons. This percentage was lower (≤ 27%) among dip vaccinated trout prior to the occurrence of vibriosis.

Monoclonal antibodies have been used in fish immunology, e.g. against serum Ig of channel catfish (Lobb and Clem, 1982), rainbow trout (DeLuca *et al.*, 1983) and carp (Secombes *et al.*, 1983) for the detection of leukocytes, such as non-specific cytotoxic cells (Evans *et al.*, 1990).

Monoclonal antibodies to rainbow trout IgM were prepared and applied in an ELISA for measuring humoral antibodies to *V. anguillarum* in trout (Thuvander *et al.*, 1990). It was found that the monoclonal anti-rainbow trout IgM antibodies discriminated seropositive fish from controls more efficiently than polyclonal rabbit anti-trout IgM antibodies.

Other assays, for example the enzyme-linked immunospot assay (ELISPOT) for quantification of antibody secretory sells (Davidson and Secombes, 1992) have been used. The optimised ELISPOT assay is at least as sensitive as the plaque assay, and is usually more sensitive (Heyman *et al.*, 1991).

CHAPTER 2.0

GENERAL MATERIALS AND METHODS

2.1 Introduction:

The following materials and methods were established 1) to prepare bacterins, 2) to produce antisera, 3) to purify antisera, 4) to develop assays for serological monitoring of the immune response of animals and also monitoring persistence of transferred antisera in fish sera, 5) to quantify a lethal dose of bacteria for challenge for the experiments undertaken in this research project. In this chapter, general materials and methods often used for all experiments are discussed. Any specific method used for a particular experiment is discussed later with that experiment.

2.2 Growth of bacteria and *Paramoeba* sp. for antigen (vaccine) preparation.

V. anguillarum:

Origin: *V. anguillarum* serotype C (01), strain 85-3954-1 isolated from rainbow trout on 18. 3. 86. The *V. anguillarum* serotype C (01), strain 85-3954-1 is a Tasmanian strain of the organism that was serologically identical with serovar C (also designated 01) supplied by Dr. Jeremy Carson (Fish Health Unit, Mt. Pleasant Laboratories, Department of Primary Industry and Fisheries, Launceston, Tasmania).

Streptococcus sp.:

Origin: *Streptococcus sp.* (biovar 1), strain No. 88.598 isolated from rainbow trout supplied by Dr. Jeremy Carson (Fish Health Unit, Mt. Pleasant Laboratories, Department of Primary Industry and Fisheries, Launceston, Tasmania).

Paramoeba sp.

Origin: Paramoeba sp. (PA-016) isolated from Atlantic salmon supplied by MS Teresa Howard (Fish Health Unit, Mt. Pleasant Laboratories, Department of Primary Industry and Fisheries, Launceston, Tasmania).

2.2.1 Antigen preparation for injection vaccination:

V. anguillarum and *Streptococcus* sp. antigens

The *Vibrio* antigen consisted of a 24-h culture of *V. anguillarum* grown initially on sheep's blood agar (BA) and then in *Vibrio* nutrient broth (Oxoid) at temperature of 25°C. *Streptococcus* sp. was grown on BA, and then inoculated in Todd-Hewitt broth (Oxoid) for 48h on a shaker at room temperature(25°C). The pH was maintained at 6.8- 7.3 with 1 N NaOH. Glucose was added after 18 h incubation (final concentration, 1.0%). Bacteria were grown to a density of approximately 10^{10} viable cells per ml. The suspensions containing *Vibrio* and *Streptococcus* cells were treated

with formalin (37% formaldehyde) to a final concentration of 0.5% v v^{-1} and left overnight at 4°C. Each suspension was washed three times [with phosphate-buffered saline(PBS, pH= 7.2)] by centrifugation (at 4000 rpm for 20 min) at 10°C and the sterility of the washed bacteria was tested by cultures on BA. Loss of bacterial viability was confirmed by lack of growth on BA before injection into fish. The washed formalin killed bacterial cells were resuspended in PBS to a density of 2 mg of cells (dry weight) in 1 ml of PBS and emulsified with an equal volume of either Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA) (Sigma) and stored at -20°C until used.

Live washed *V. anguillarum* and *Streptococcus* sp. cells were also sonicated (3x1 to 3x3 cycles or disrupted in a French press [(Elizabeth Macarthur Agricultural Institute (EMAI) for *Streptococcus* cells)], centrifuged and filtered through 0.2 μ filter for coating the ELISA plates. Protein concentration of the supernatant was determined by a Commassie Blue dye binding assay (for *V. anguillarum* antigen) (Bradford, 1976) and a Lancer Microprotein rapid stat diagnostic kit (for *Streptococcus* sp. antigen).

Paramoeba sp. antigens:

Paramoeba sp. was maintained on a bacterial lawn of *Pseudomonas maltophilia* grown on malt yeast extract seawater agar. *Paramoeba* were harvested from the plate and suspended in sterile seawater and homogenised. The harvested *Paramoeba* were used for preparing sonicated antigen. Both *Paramoeba* cells and sonicated cells were treated with formalin (37% formaldehyde) to a concentration of 0.5% v v^{-1} and emulsified with either an equal volume of FCA or FIA for vaccination as explained previously.

Live *Paramoeba* sp. was also sonicated (3x1 to 3x3 cycles) and filtered through 0.2 μ filter for immunisation of fish and coating the ELISA plates. The protein content was then measured using a Lancer Microprotein rapid stat diagnostic kit.

2.2.2 **Antigen Preparation for immersion vaccination:**

V. anguillarum were grown in vibrio nutrient broth (Oxoid) while *Streptococcus* sp. was grown on BA and then inoculated in Todd-Hewitt broth (Oxoid) at a temperature of 25°C for 48 h. Both organisms were grown to a density of approximately 10^{10} viable cells ml^{-1} as explained in the previous section treated with formalin (37% formaldehyde) to a final concentration of 0.5% v v^{-1} and stored overnight at 4°C. The formalised cultures were kept at +4°C until used.

2.3 **Animals** :

Experimental animals included sheep, rabbits and fish: Sheep were maintained at the Department of Primary Industry and Fisheries, Mt. Pleasant Laboratory, Launceston, Tasmania. New Zealand white rabbits (approximately 2 kg) were maintained in the animal laboratory unit at Elizabeth Macarthur Agricultural Institute

(EMAI), NSW and Animal House Unit, University of Tasmania, Hobart campus. Fish were rainbow trout (*Oncorhynchus mykiss*, Walbaum) and Atlantic salmon (*Salmo salar* L) (weight range of 50 - 620 g). Fish were maintained in freshwater (rainbow trout) or seawater (Atlantic salmon at the time of exposure to *Paramoeba*) in temperature controlled tanks at the Aquaculture Key Centre, Department of Aquaculture, University of Tasmania.

2.4 **Production of antiserum.**

Two sheep, two rabbits, fifty large rainbow trout and forty Atlantic salmon were used for producing antisera against *V. anguillarum* or *Streptococcus* sp. The same number of animals were used for anti-*Streptococcus* sp. antisera production. Antisera production against *Paramoeba* sp. is described in chapter 5.0.

Sheep were injected subcutaneously in the cervical area with a 2 ml dose (2 mg dry weight of formalin killed cells) of vaccine containing FCA at week 0, and 2 ml of the same vaccine in FIA at week 4. Animals were bled from the jugular vein at the end of week 4 before administration of the booster dose in order to assess their humoral antibody response to the antigen. At the end of week 6 blood samples were collected and sheep were euthanised. Serum was collected after allowing the blood to clot overnight at 4°C and centrifuged (1400 g) and was stored at -20°C.

Similarly, rabbits were injected subcutaneously with antigens but were bled by incising the ear veins.

Fish maintained in 4000 L fibreglass tanks at a temperature of 15°C were used for raising antisera. All fish were starved 12 hours then anaesthetised for experimental procedures with 40 mg l^{-1} benzocaine (10% w v^{-1} ethyl-4-aminobenzoate in ethanol, or acetone). Fish were anaesthetised in a 20 L fish bin with aeration and a flow through aerated 450 L recovery bin was used for recovery of the anaesthetised fish. A volume of 0.1 ml dose (0.1 mg dry weight of formalin killed cells) of each prepared vaccine containing adjuvant was injected intraperitoneally (midway between the pelvic fin and the anus) at week 0 and at week 4 respectively. Fish were bled via the caudal vein. Unless stated otherwise, sera were all tested by ELISA prior to the immunisation and found to be free of specific antibodies.

2.5 **Purification of antisera:**

2.5.1 **Ammonium sulphate (Amm.Sul) precipitation of antisera**

In order to precipitate antibodies, each serum was centrifuged at 3000g for 30 min and supernatant was used for the next step. While the serum was being stirred gently, saturated ammonium sulphate solution was slowly added to 50% v v^{-1} and the mixture was transferred to 4°C overnight. After centrifugation of the precipitate at 3000g for 30 min, the supernatant was carefully discarded and the pellet was

resuspended in 0.3-0.5 volumes of the starting volume in PBS (Harlow and Lane, 1988).

The solution containing antibodies was placed into dialysis tubing and dialysed versus 25 mM tris pH 7.2, 0.15 M NaCl overnight. Samples were removed from the tubing and centrifuged to remove any remaining debris. Aliquots containing antibodies were stored in the presence of 0.02% sodium azide at -20°. Sera of sheep, rabbit and fish were precipitated with this method for further use.

2.5.2 **Affinity purification of sheep immune serum on protein G-sepharose:**

Protein G-sepharose ®4 Fast Flow (Pharmacia®) (5 ml, preswollen in 20% ethanol) was used for purification of sheep antisera according to the method established by Dr Richard Whittington (EMAI). The swollen gel was poured smoothly into an emptied PD-10 column (Pharmacia) and packed using 150 ml of starting buffer. Serum (1-2 ml) was centrifuged at 3000 g for 10 min in a microcentrifuge. The supernatant was mixed with an equal volume of starting buffer [(25 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 0.01% sodium azide) (all reagents' formula in appendix V) , loaded onto the column and washed through with 25 ml of starting buffer at a flow rate of 10 ml hr^{-1}. Immunoglobulins (Ig) were eluted with 30 ml of 0.1 M glycine-HCl pH 3, 0.15 M NaCl, at a flow rate of 30 ml/hr. Fractions of 2.5 ml were collected using a Gilson model 202 fraction collector and immediately neutralised by the addition of 35 µl of 2 M Tris. Pooled fractions were dialysed overnight against starting buffer, and concentrated by dialysis against Aquacide II (Calbiochem) to the original volume of serum. The yield of Ig (dry weight) was 9 mg ml^{-1}. Resulting antisera was evaluated for specific activity by ELISA. Purified Ig were stored at -20°C for further use.

Protein content was estimated by the Microprotein rapid stat kit (Lancer, Oxford Labware, St. Louis. MO.63103 USA). The protein content of pooled affinity protein-G purified sheep anti-*V. anguillarum* was 9.4 mg ml^{-1}.

2.5.3 **Affinity purification of rabbit immune serum Ig on protein A-sepharose:**

Protein A-sepharose CL-4B (Pharmacia®) (1.5 g) was swollen in 20 ml of starting buffer , for 15-20 min and poured into an emptied PD-10 column (Pharmacia). The remainder of the procedure was as described above for protein G.

The yield of Ig (dry weight) was 14 mg ml^{-1}. Resulting antisera was evaluated for specific activity by ELISA and SDS-Page.

Gels (protein G, A-sepharose) were then regenerated using Tris regeneration buffer (0.1 M Tris-HCl, pH 8.5, 0.5 M NaCl), acetate regeneration buffer (0.1 M Na acetate pH 4.5, 0.5 M NaCl) and starting buffer.

2.5.4 Purification of fish immune serum Ig on washed formalin inactivated *Vibrio anguillarum* cells.

The following method for purification of fish immune serum was attempted using formalin killed *V. anguillarum* cells. Fish AVA serum was precipitated with ammonium sulphate and concentrated as described before. Several dilutions from 1 to 500 µl ml^{-1} were prepared. Washed formalin-killed *V. anguillarum* cells were then added, mixed and left overnight at 4°C. The suspension was centrifuged (1400 x g) and the elution buffer (glycine-HCl, pH 3) was added and mixed. The suspension was then centrifuged (1400 x g) and the supernatant was neutralised and concentrated.

Several efforts were undertaken not only to use different concentration of antibodies as explained, but also several dilution of washed killed bacteria with the same elution buffer.

ELISA was run to verify fish Ig activity. All the ELISAs were negative therefore, this method of fish serum purification was unsuccessful.

2.5.5 Assessment of the purity of ammonium sulphate precipitated and affinity purified serum samples by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Different sera were assessed by SDS-PAGE (Laemmli, 1970) in an 8 cm, 12% resolving, 4 % stacking gel. The gel was run at 60 mA and 138 volts for one and a half hour. Protein bands were stained with 0.2% Commassie brilliant blue for 50 minutes. A volume of 15 µl of each serum sample with a 10 µg protein content was assessed (Plate 2.5.5).

SDS-Page for sheep antisera.

For sheep:

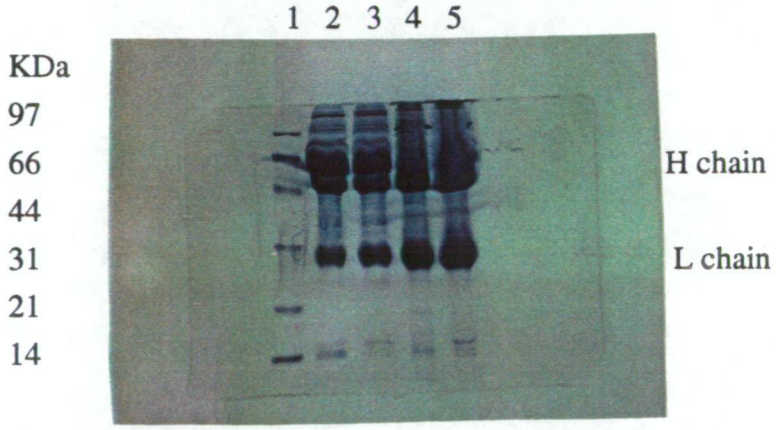
1- MW standards.

2- Sheep whole non-immune serum (15 µl of 1:20 dilution of serum).

3- Sheep whole serum immune serum (15 µl of 1:20 dilution of serum).

4- Sheep ammonium sulphate non-immune serum (10 µg protein).

5- Sheep ammonium sulphate immune serum (10 µg protein).



Low molecular weight
protein ≤10 KD
dalton

SDS-Page for rabbit antisera

1- MW standards.

2- Rabbit whole non-immune serum (15 µl of 1:20 dilution).

3- Rabbit whole immune serum (15 µl of 1:20 dilution).

4- Rabbit ammonium sulphate non-immune serum (10 µg protein).

5- Rabbit ammonium sulphate immune serum (10 µg protein).

6- Rabbit affinity immune serum (10 µg protein).

7- Rabbit affinity immune serum (10 µg protein).

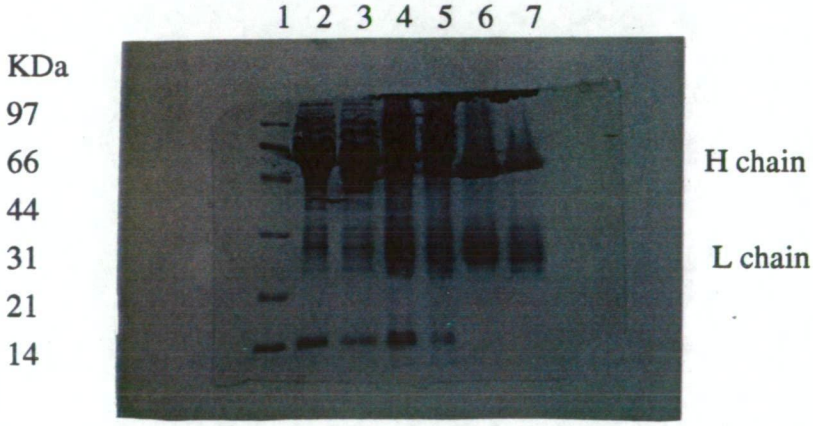


Plate 2.5.5: SDS-Page of sheep and rabbit sera

2.6 Enzyme-linked immunosorbent assay (ELISA)

Detection of antibodies in sera :

The test used to detect anti-*V. anguillarum*, anti-*Streptococcus* sp. and anti-*Paramoeba* sp. antibodies in immune sera, and sera of passively immunised fish, was a specific indirect enzyme-linked immunosorbent assay developed by Dr. Richard Whittington (EMAI).

Solid phase: The microtest plates used were flat bottom polystyrene ELISA microtitration plates Linbro/ Titertek, (CAT.NO.76-381-04), Flow Laboratories, Inc. USA.

Antigens:

To perform indirect ELISA, whole cell bacteria (WCB) and soluble antigen (SA) were used for coating the plates.

1-Whole cell antigens

Bacteria were grown on sheep blood agar for 24 h and harvested from the plate by flushing with sterile saline. Harvested cells were washed twice in sterile saline (3500 rpm, 20 min.) and the pellet was resuspended in a known volume of PBS.

The absorbance of the washed bacteria was measured at 650 nm and the bacteria were resuspend in a volume to achieve an A650 of 4 in both TSGM and TS methanol (ELISA reagents, appendix III).

2- Soluble antigens

Soluble antigens were prepared by sonication of live washed bacteria as described in section 2.2.1. Several 1 ml solution of the antigens was prepared, diluted 1: 10 in TSGM and was stored at -20°C.

General ELISA method:

Microtitre plates were coated with 100 µl/well of WCB antigen diluted 1: 750-1: 1000 in TS methanol and were centrifuged for 10 min at 2500 rpm at 10°C. Alternatively, plates were coated with 1-5 µg soluble antigen diluted in borate coating buffer, pH 8.4 (100 mM boric acid, 25 mM disodium tetraborate, 75 mM NaCl) incubated 16 h at 4°C. The antigen was then flicked off and remaining free binding sites were blocked with 100 µl well^{-1} of 1% w v^{-1} gelatin (Oxoid) in PBS+ 0.05% Tween 20 (PBST) for 30 min at room temperature (RT), then washed 5 times by distilled water+ 0.05% Tween (DWT) using Titertek® microplate washer 120 (Flow Laboratories, UK). Serially diluted test sera (primary antibody), were added (100 µl well^{-1}) to the wells and incubated for 90 min at RT. Duplicate wells were used for each serum sample.

After washing with DWT, either conjugate (labelled anti-species antibody) (sheep ELISA; KPL® anti-sheep-HRP and rabbit ELISA; Dako® anti-rabbit-HRP) diluted 1: 2000, or mouse monoclonal antibody (mAb) anti-trout (fish ELISA) (Deluca *et al.*, 1983), supplied by Dr. Richard Whittington, EMAI as secondary antibody diluted 1: 1000 (in PBS containing 0.5% Tween and 0.1% gelatin) was added and incubated for another 90 min at RT.

Fish ELISA required one more step for attachment of specific conjugate (Dako® rabbit anti-mouse) to monoclonal antibody and incubated for 90 min at RT. After washing, 100 µl well^{-1} of 2,2'-azino-bis (3-ethyl benzen thiazoline-6-sulfonic acid) Diammonium salt (ABTS) in 100 mM citrate phosphate pH 4.2, 2.5 mM hydrogen peroxide was added (Fig 2.6.1.1). The reaction was stopped after 20 min at RT by the addition of 50 µl of 0.015 sodium azide in 0.1 M citric acid and optical density (O.D) was measured at 405 nm using an automated microplate reader model EL 309 (Bio-Tek Instruments Inc., USA). The obtained optical densities were compared with the developed colour in wells visually for any possible variation due to edge effect. A negative and positive control serum was included on each plate.

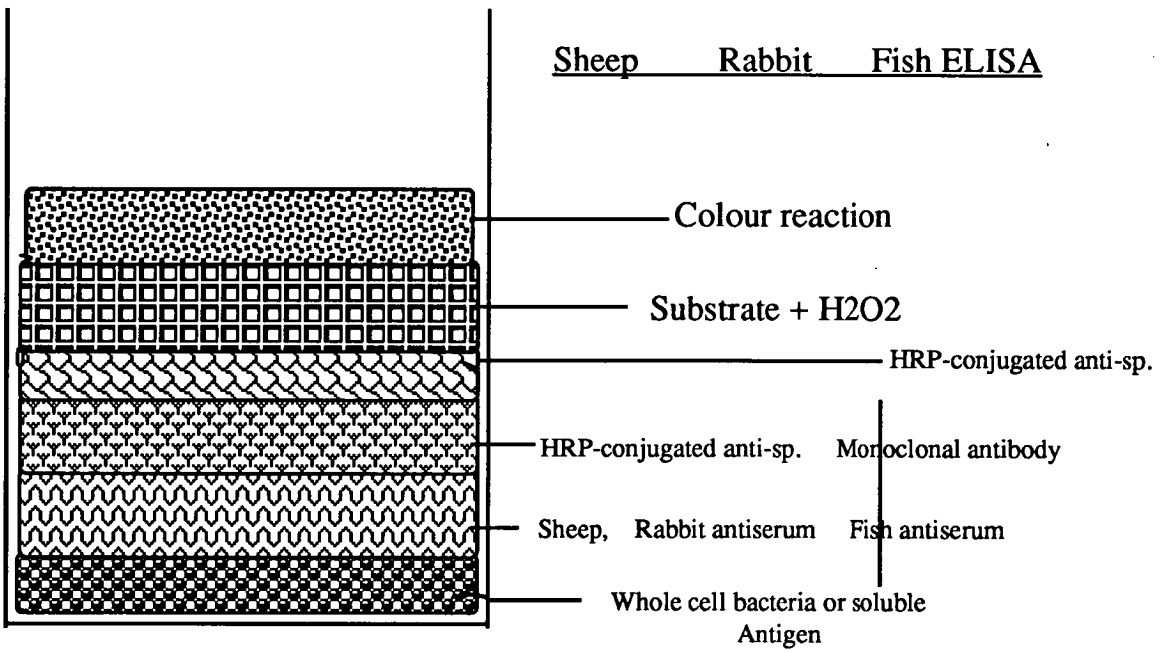


Figure 2.6.1.1: Configuration of sheep, rabbit and fish ELISAs.

Control positive and negative sera were included in triplicate in every plate. These sera originated from stock fish before immunisation. Positive control sera was the pooled sera of trout that had been repeatedly immunised with formalin-killed bacterins. The known serum containing a final dilution equivalent to the final dilution of the test sera was used in 4 dilutions; highly positive, medium positive, low positive and negative. The diluent for control was negative sera. This was to ensure that the

concentration of background proteins in the sera (test sera) and controls (control sera) are similar. By use of these control, it was possible to correct the optical densities (explained later).

In this test, antibody (serum) dilution was variable and antigen and marked antibodies (conjugate, monoclonal) were usually held constant. The pH of all diluents for serum, monoclonal antibody and conjugate in both sheep and rabbit ELISA were adjusted to 7.2-7.4 and in fish ELISA adjusted to 8.0. Due to the small amount of fish blood and therefore small amount of sera instead of using 100 µl well^{-1} antigen, serum and etc. 50 µl well^{-1} was used (in fish ELISA). The optimal concentration of antigen, conjugate and monoclonal antibody were titrated upon their use in ELISA before running the test (explained later).

A positive serological result was defined as an O.D greater than mean+ 2 S.D of the OD of control fish sampled at the same time.

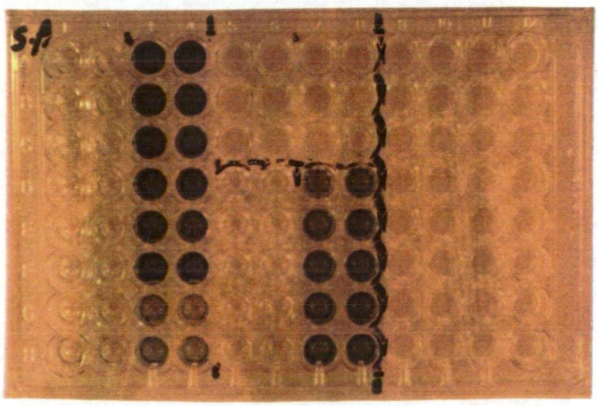


Plate 2.6.1: ELISA plate showing colour reactions at different dilutions of antiserum

2.6.1 **ELISA for detecting sheep anti-*V. anguillarum* antibodies**:

A standard, indirect, enzyme-linked, immunosorbent assay was used to detect antibodies against *V. anguillarum*. This procedure was applicable to detect anti-*Vibrio* antibodies (AVA) both in sheep serum and in sera of fish which were passively immunised with immune sheep serum. The reagents were rabbit antibody against sheep Ig, conjugated to HRP and ABTS chromogen with hydrogen peroxide as substrate for the enzyme. In this test the sheep non-immune and immune AVA were examined.

Procedure for running ELISA for sheep anti-*Vibrio* antibodies:

In this test AVA in sheep serum (pooled sheep sera) were detected. All washes were distilled water+ 0.05% v v $^{-1}$ Tween 20. Diluent (PBSTG) for serum and conjugate was at pH 7.2-7.4. Samples were run in duplicate on each plate (summarised ELISA procedure is in appendix IV). No edge effect was observed after reading ELISA plates.

A number of parameters required optimisation since the method was being transferred from EMAI where different equipment and apparatus was used, as well as different batches of reagents. Sheep antibody response to *V. anguillarum* antigens using whole cell bacteria (constant dilution 1: 750), conjugate (constant dilution 1: 2000) and variable level of antibody is shown in Figure 2.6.1.2.

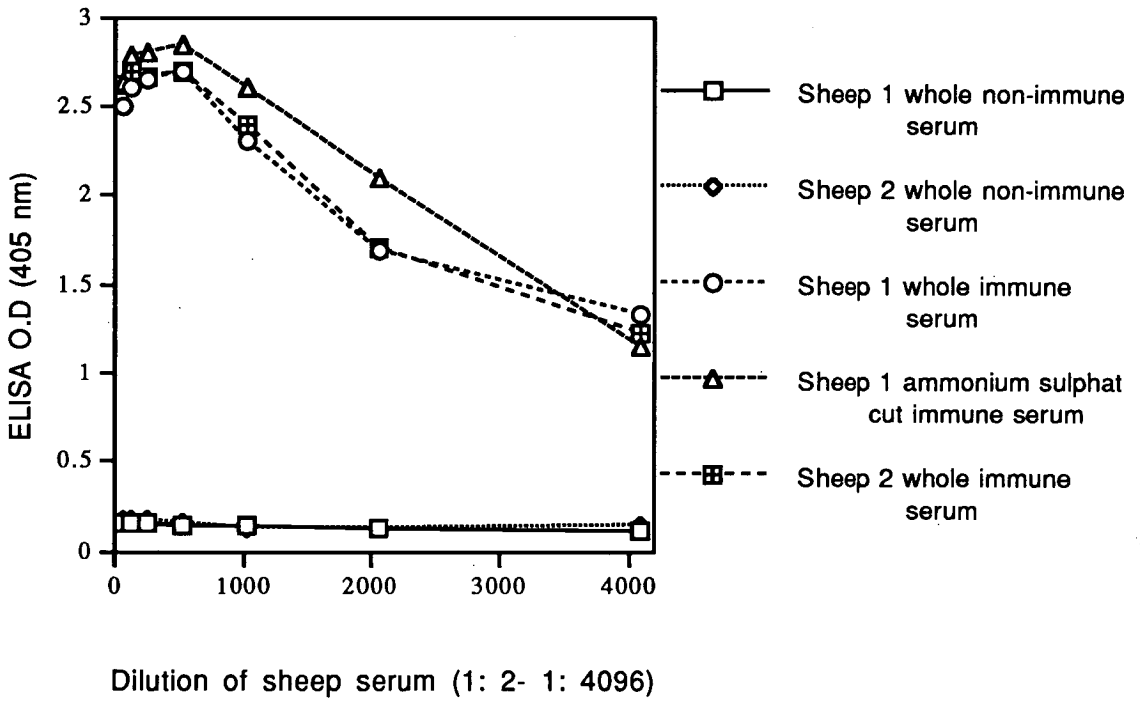


Figure 2.6.1.2: Optical density vs dilutions of sheep serum (sheep non-immune and immune sera)

The results show that ammonium sulphate cut serum had higher O.D suggesting higher antibody level of the precipitated immune serum.

Signal noise ratio (without any unite) was calculated by dividing optical density of immune serum (mean of the duplicate wells) by O.D of non-immune serum (mean of the duplicate wells). Therefore the higher the signal noise the higher ELISA activity at that particular dilution of sera etc. Signal noise of sheep sera at different dilutions showed that an optimum dilution of 250 (Figure 2.6.1.3).

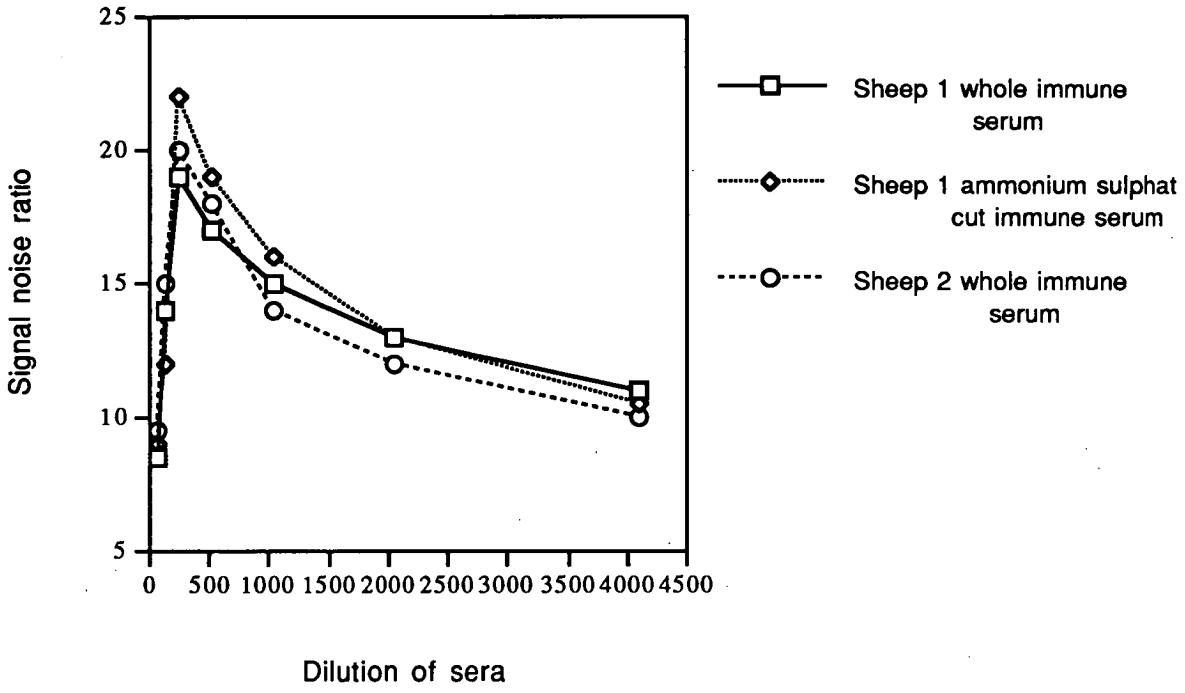


Figure 2.6.1.3: Signal noise vs dilution of sheep AVA

Regarding optimisation of antigens, different dilutions of antigen (WCB) with constant dilution of immune serum were used.

The results comparing the binding activity of whole cell bacteria and soluble antigens with 1: 200 dilution of serum and different dilution of conjugate (conjugate titration) are shown in figure 2.6.1.4.

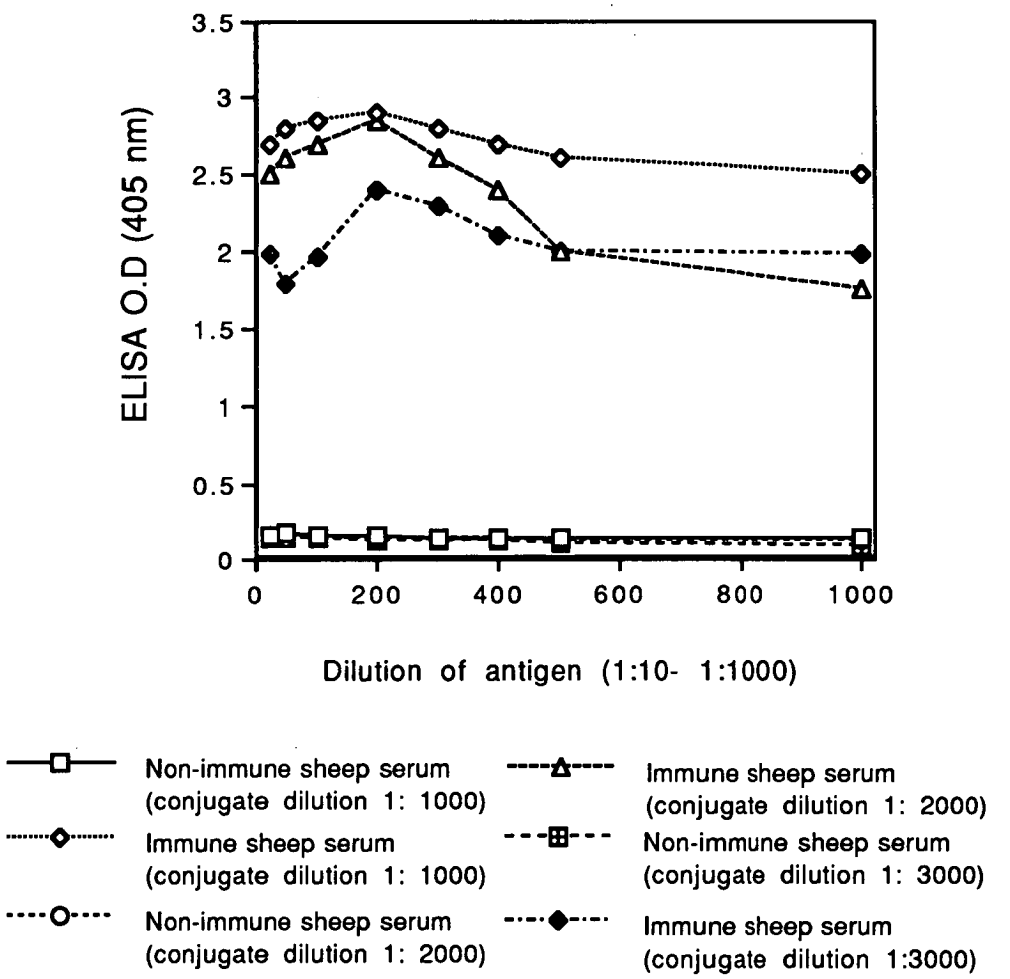


Figure 2.6.1.4: Optical density vs dilution of bacterial antigen (WCB) in different dilutions of conjugate

Signal noise of the dilutions of antigen (WCB) in different dilution of conjugate and constant dilution of sheep sera (1: 200) is shown in figure 2.6.1.5.

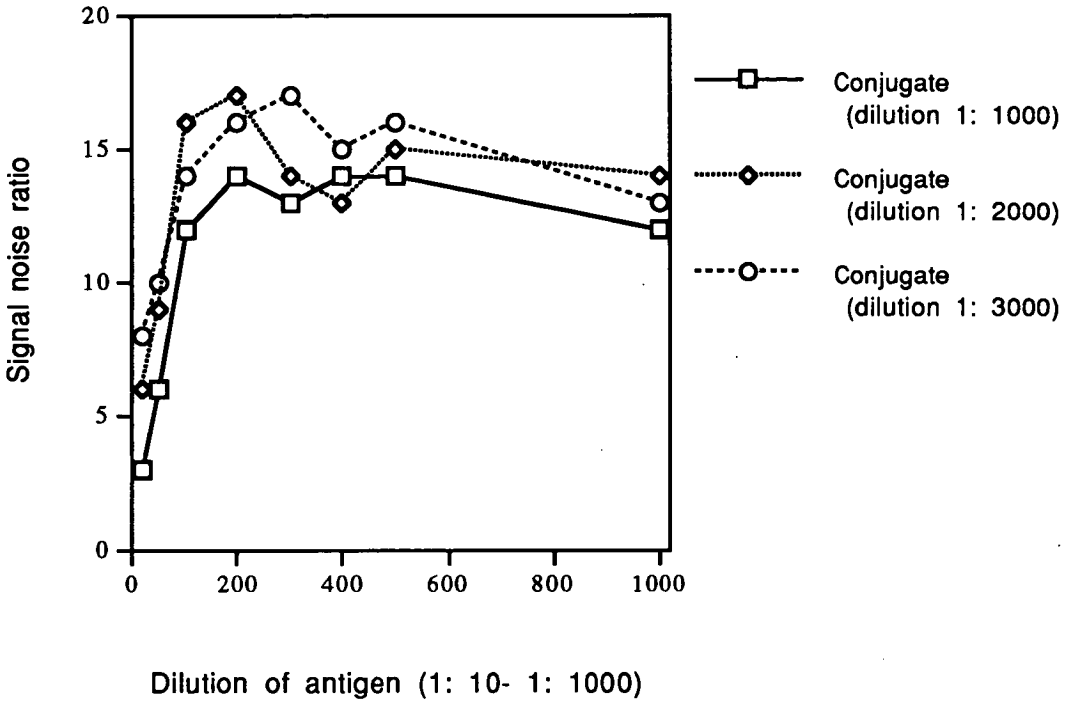


Figure 2.6.1.5: Signal noise vs different dilutions of antigen (WCB) and conjugate

The results showed that dilution 1: 200 of whole cell bacteria (WCB) had the best binding property to the plate and the dilution of 1:2000 of conjugate demonstrated a higher activity in sheep ELISA.

In order to optimise the concentration of soluble antigen, different concentrations of soluble antigen and conjugate with constant dilution of sheep sera (1: 200) were used (Figure 2.6.1.6).

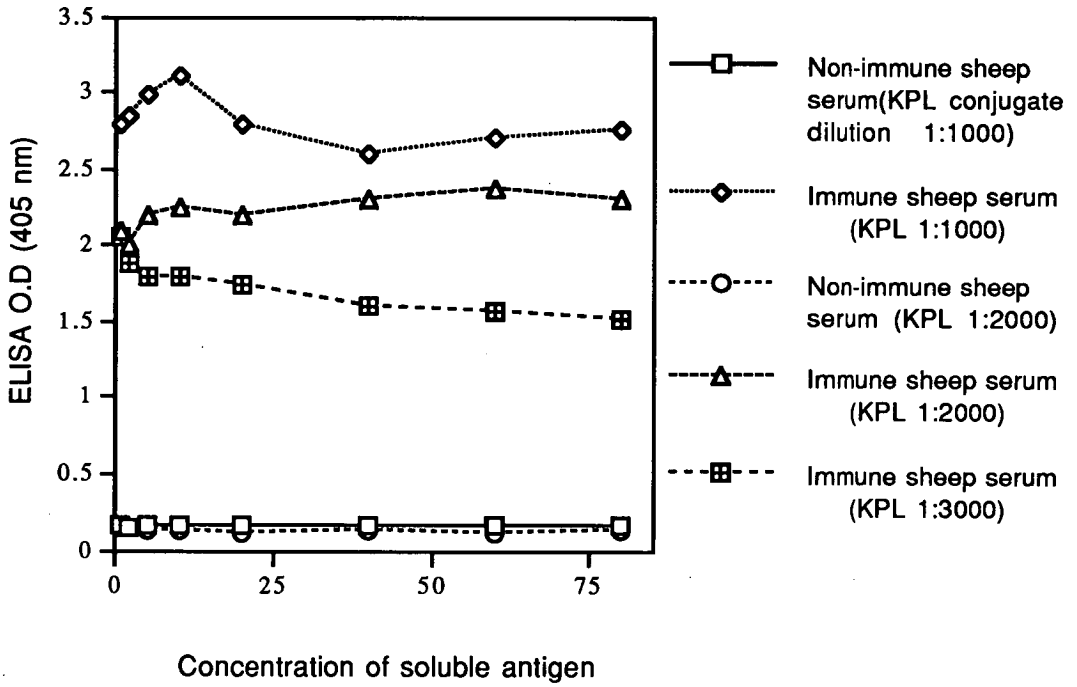


Figure 2.6.1.6: Optical density vs concentration ($\mu g\ ml^{-1}$) of soluble antigen in different dilution of conjugate

Dilution of antigen in 1-10 $\mu g\ ml^{-1}$ had a high binding property to the ELISA microplate and conjugate at the dilution of 1: 1000 showed the highest activity.

In comparing the binding activity of two types of antigens (WCB and soluble antigens), both showed high binding properties. However, soluble antigen showed higher activity in detecting sheep AVA.

Prozone (presence of inhibitors of antigen-antibody binding which are diluted out, resulting in higher signals at higher dilutions of serum, up to the point where further dilution reduces the antibody enough to suppress the signal) was observed in undiluted and low diluted sera. Therefore, in all sheep ELISAs, dilution of 1: 200 of either non-immune and immune sheep sera was used.

It can be deduced from the results that different biological reagents which were used in ELISA had to be titrated in order to use the most active amount of the reagents before testing the test sera.

2.6.2 ELISA for detecting rabbit anti-*V. anguillarum* antibodies:

ELISA was run for detecting AVA in rabbits sera (pooled rabbit sera). Two non-immune and two immune rabbit sera were tested as well as those ammonium sulphate treated sera. A standard, indirect, ELISA was employed. Reagents were Dako swine

against rabbit Ig, conjugated to horse-radish peroxidase (dilution of 1: 250) and ABTS chromogen with hydrogen peroxide as substrate for the enzyme. In this test the non-immune and immune sera of sheep were examined for their AVA levels. In addition, ammonium sulphate purified immunoglobulin was also tested (summarised ELISA procedure for detecting rabbit antibodies in appendix IV). No edge effect was observed after reading ELISA plates.

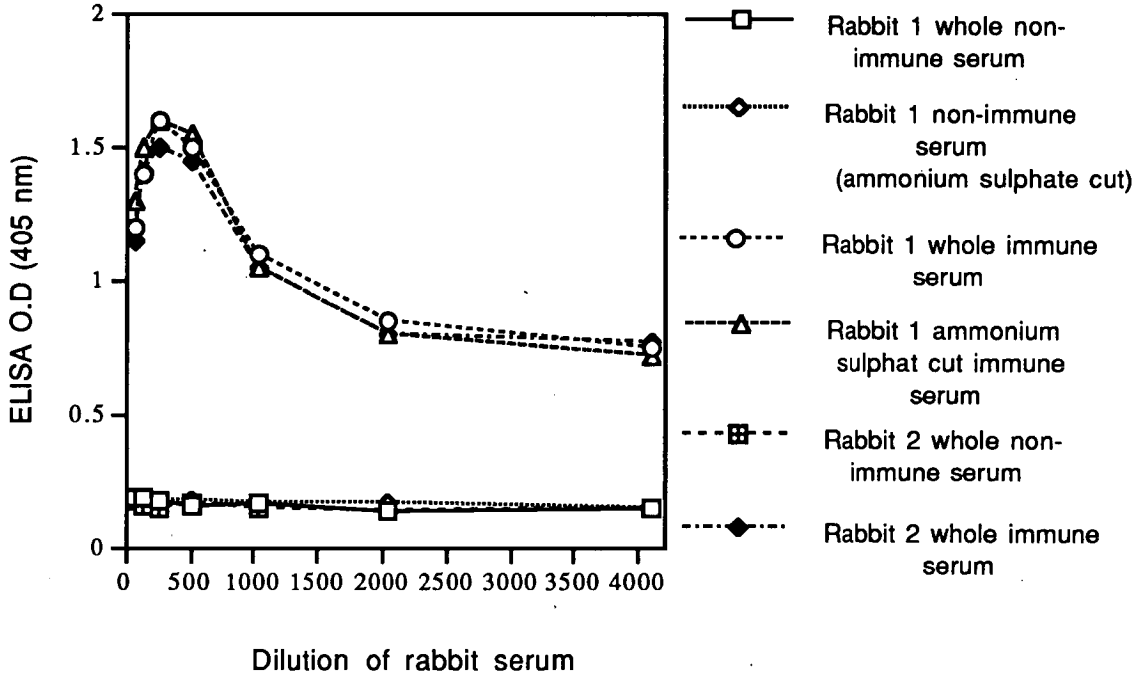


Figure 2.6.2: Optical density vs dilution of rabbit serum.

In rabbit ELISA, all immune sera showed a great deal of anti-*Vibrio* antibodies. However the amount were not as high as sheep's. It seems that sheep responses to *V. anguillarum* were higher than that of rabbit. The prozone effect was observed in whole and low diluted sera.

Optimum dilutions of conjugate, antigen (WCB) and soluble antigen in this ELISA were 1:500 and 1:100 and concentration of 5 μg protein respectively. Soluble antigen showed higher activity in detecting AVA when compared with whole cell antigen. Signal noise was also calculated to find out the best concentration of each reagents as those of sheep ELISA (as described previously). Dilution of 1: 200 of rabbit sera showed the best activity. Therefore, in all rabbit ELISAs these dilutions were used. ELISA O.D results, maximum, minimum and cut-off values of fish antibody responses after i.p. injection of 1 mg (dry weight) formalin killed *Vibrio* cells are summarised in appendix VI.

2.6.3 ELISA for detecting fish antibodies

2.6.3.1 ELISA for detecting fish antibodies to *V. anguillarum* antigens:

ELISA was run for testing non-immune and immune sera (pooled fish sera) produced in the Aquaculture Key Centre and also those from EMAI. The specific reagents were mouse monoclonal antibody against trout IgM heavy chain, rabbit antibody against mouse IgG conjugated to HRP and ABTS chromogen with hydrogen peroxide as substrate for the enzyme.

In this test the non-immune and immune sera of fish were examined for their anti-*Vibrio* antibodies levels. Mouse mAb (anti-trout IgM) was added to attach to anti-*Vibrio* antibodies and then conjugate (Dako rabbit anti mouse-HRP is added to react with it (summarised procedure in appendix IV).

The amount of antiserum and reagent were reduced to half amount because of relatively small volume of antisera taken from fish. ELISA for detecting fish response to *Vibrio* antigen was set up using dilutions of antigen (WCB), 1:1000 mAb, 1:10 in PBSTG and conjugate dilution in 1:750.

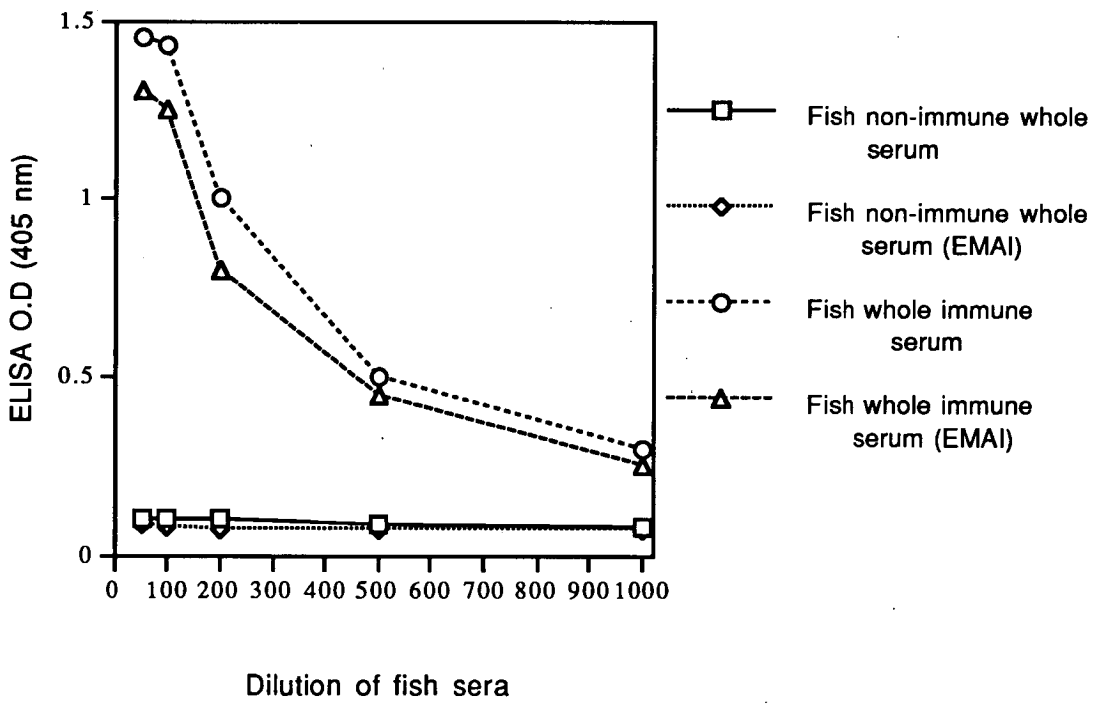


Figure 2.6.3.1: Optical density vs dilution of fish sera

The results of this test showed that fish had responded to *V. anguillarum.* markedly at a dilution of 1: 100. The titres in dilution up to 1: 1000 still showed significant antibody level. Optimisation of other parameters was also carried out to determine the best dilutions as described previously. Using whole cell antigens (1: 750) and soluble antigen 10 $\mu g\ ml^{-1}$ showed that whole cell antigen resulted in higher detection of fish antibodies. Therefore, for coating the plates in fish ELISAs, whole cell antigen was used.

2.6.3.2 ELISA method for detecting fish antibodies to heterologous sheep and rabbit antisera:

Microtitre plates were coated with 50 µl of 1 µg protein ml^{-1} either ammonium sulphate purified sheep or rabbit AVA (diluted in borate coating buffer). Plates were sealed and incubated at + 4°C overnight. Plates then were washed 5 times with DWT and remaining free binding sites were blocked with 100 ml $well^{-1}$ of 1% W/V gelatin (Oxoid) in PBS + 0.05% Tween 20 (PBST) for 30 min at room temperature (RT).

Plates were washed 5 times in DWT and 1: 100 dilution of fish sera (collected from fish after injection of sheep and rabbit AVA) were added (50 µl $well^{-1}$) to the wells and incubated for 90 min at RT. Duplicate wells were used for each serum sample. After washing in DWT, mouse monoclonal antibody anti-trout was added and plates were incubated for 90 min at RT. Rabbit anti-mouse-HRP was then added and plates were incubated for another 90 min at RT. After washing, 50 µl $well^{-1}$ of ABTS in 100 mM citrate phosphate pH 4.2, 2.5 mM hydrogen peroxide was placed into the wells. The reaction was stopped after 20 min at RT by the addition of 50 µl of 0.015 sodium azide in 0.1 M citric acid and optical density (O.D) was measured at 405 nm.

In order to confirm binding of the sheep/rabbit Ig to the microtitre plates, control wells were included in the last column in each plate in duplicate. Fish serum was not added to these wells, which were reacted directly with rabbit anti-sheep-HRP conjugate (for sheep plate) and swine anti-rabbit-HRP (for rabbit plate), for 90 min at RT. After washing, 50 µl $well^{-1}$ of ABTS was added and the assay continued as above. A positive result in these wells confirmed the binding of either sheep or rabbit Ig.

2.6.4 ELISA for detecting anti-*Streptococcus* sp. antibodies (ASA):

A standard, indirect, enzyme-linked, immunosorbent assay was established for detecting anti-streptococcal antibodies in sera. Optimising of reagents (antigen, conjugate and monoclonal antibodies) was carried out to establish a sensitive ELISA as described in the *Vibrio* ELISA. In this test non-immune and immune serum of sheep rabbit and fish against *Streptococcus* sp. were examined.

Antigen preparation:

The bacteria were grown on blood agar for 48 h and harvested from the plate by flushing with sterile saline or from broth by centrifuging. Harvested cells were washed twice in sterile saline(4000 rpm, 20 min) and the pellet was resuspended in a known volume of sterile PBS.

1- Whole cell bacteria: The absorbance of washed bacteria was measured at 650 nm and the solution was resuspend in a known volume of TS-methanol to achieve an absorbance (650 nm) of 4 (neat antigen) as described in section 2.6. Plates were coated with this antigen from non-diluted to dilution of 1: 750. Results of the ELISA

using sheep anti-streptococcal antibodies in order to optimise the antigen is shown in figure 2.6.4.1.

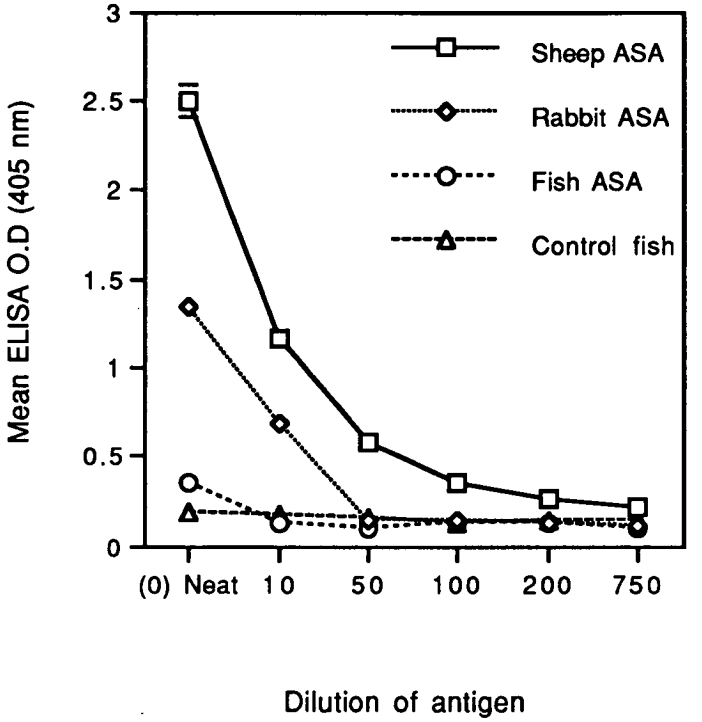


Figure 2.6.4.1: Optical density vs dilution of whole cell *Streptococcus* sp. antigen

The results showed that neat WCB had the best binding property to antibodies in the serum. Nevertheless, this dilution was not always consistent. This is probably because anti-*Streptococcus* sp. antibodies (ASA) does not react enough to the antigen because of low affinity. Therefore, this assay needs a high concentration of the whole cell bacterial antigen in order to monitor the specific ASA.

2- Soluble antigen: Bacterial lawns were harvested, washed and centrifuged in the same manner as described above for whole cell bacteria. The pellet was suspended in a volume of PBS and disrupted by French press as described in section 2.2.1.

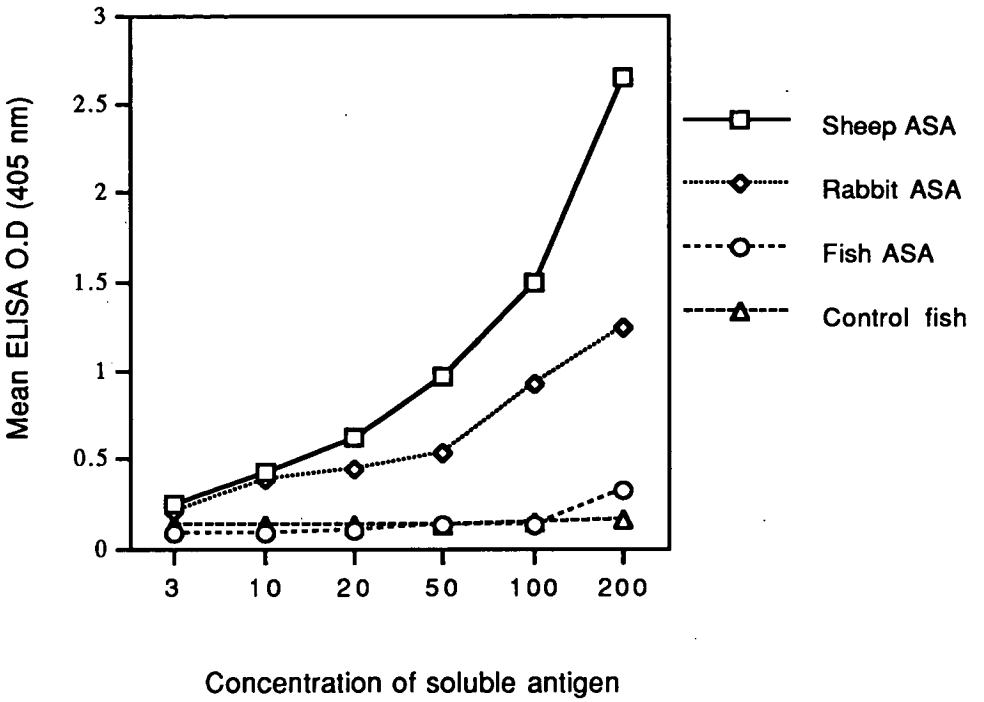


Figure 2.6.4.2: Optical density vs concentration of soluble antigen (μg ml^{-1}) with conjugate dilution of 1: 1,500

Higher optical density was observed at a concentration of 200 μg ml^{-1} of soluble antigen (Figure 2.6.4.2). This suggests that the ASA show high affinity to the sonicated antigen as it is likely that the immunogenic antigen was relatively enriched in the sonicated antigen relative to the whole cell antigen. Also, the sonicated antigen may have bound to the plate more efficiently. The results suggest that both WCB and soluble antigen have very poor binding to the plates. In subsequent assays a high concentration of antigen was used

Also ELISAs were run using high absorbance plates (NUNC Maxisorb) with both WCB and sonicated *Streptococcus* sp in different dilutions. There was no significant difference between Linbro ELISA plates and NUNC Maxisorb plates. Covalent plates may be required.

ELISA for sheep, rabbit and fish anti-*Streptococcus* sp. antibodies was run to optimise different dilution of reagents as described for the *Vibrio* ELISA.

Anti-*Streptococcus* sp. antibodies activity was determined in sheep, rabbit and fish sera immunised intraperitoneally by streptococcal vaccine. Non-immune and immune sera were compared. Microplate was already coated with the neat (absorbance = 4.0) whole cell antigen. Conjugate was used at a dilution of 1: 1,500 after optimisation in a series of *Streptococcus* sp. ELISA experiments. No edge effect was observed after reading ELISA plates. Optical densities were reported for each well together with the mean of these for each sample. These results are shown in figure 2.6.4.3.

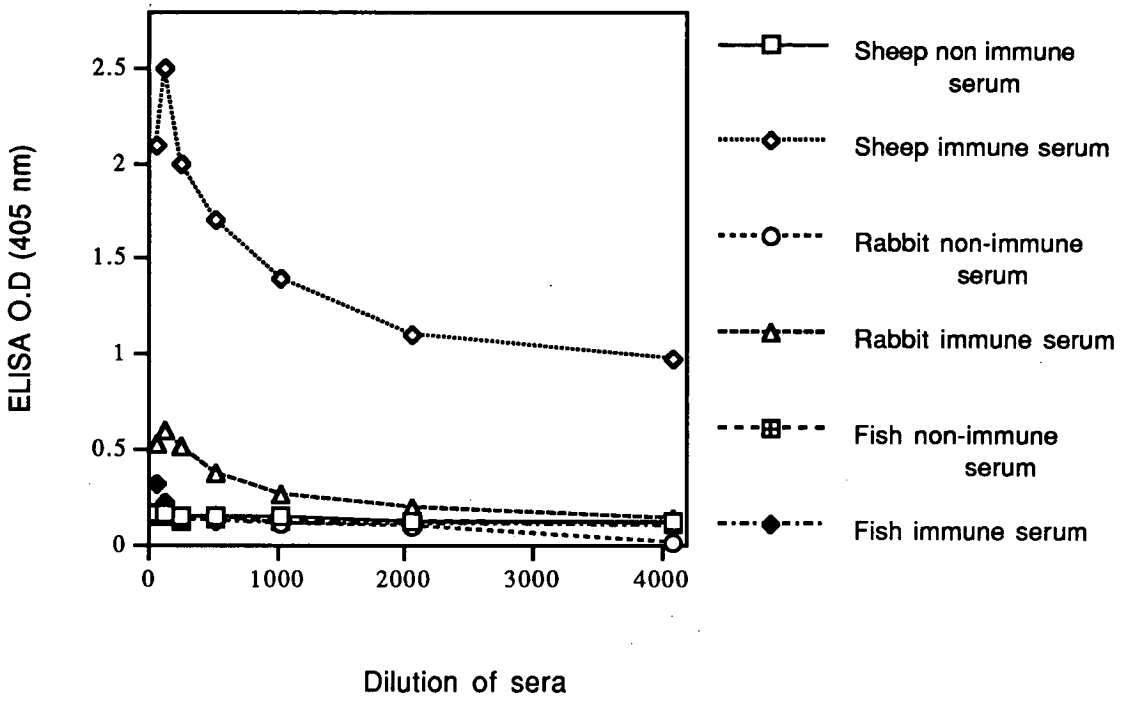


Figure 2.6.4.3: Optical density vs dilution of sheep, rabbit and fish anti-*Streptococcus* sp. sera

Signal noise ratio was calculated as it is described for *Vibrio* ELISA in order to find out the best dilution of either antiserum, conjugate and monoclonal antibodies (The graphs for these titrations are not shown because of their similarities to what was done for *Vibrio* ELISAs). Anti-*Streptococcus* sp. antisera of sheep, rabbit and fish 1: 200, 1: 200 and 1: 100 respectively; whole cell antigen as neat for all ELISAs; monoclonal antibody (1: 10 dilution, fish ELISA); conjugates, rabbit anti-sheep HRP 1: 2000, swine anti-rabbit-HRP 1:1000 and rabbit anti-mouse 1: 1500 had the highest signal noises. ELISA O.D results, maximum, minimum and cut-off values of fish antibody responses after i.p. injection of 1 mg (dry weight) formalin killed *Steptococcus* sp. cells in 50% v v^{-1} FCA are summarised in appendix VI.

2.6.5 ELISA for detecting anti-*Paramoeba* sp. antibodies :

ELISA was run with sonicated *Paramoeba* sp. as antigen and using absorbed anti-*Paramoeba* sp. antiserum to determine the animals response to the organism. In this ELISA, all parameters were titrated in order to optimize the test as described previously for other ELISAs to find out the best dilution of each reagent. The ELISA used for detecting antibodies against *Paramoeba* sp. was a modification of the ELISA described by (Bryant *et al.*, 1995). The test sera was absorbed both with sonicated antigen and live *P. maltophilia* cells.

Preparation of sonicated *Paramoeba* sp. antigen:

The method used for preparation of sonicated *Paramoeba* vaccine was as described in section 2.2.1.

Preparation of bacterial antigen:

Soluble antigen was prepared by washing, resuspending in PBS and sonicating *P. maltophilia* cells by 3 x 3 minute cycles. Bacterial sonicate was filtered (0.2μm) and its protein concentration was determined and stored at -20°C for further use.

Serum absorption:

Paramoeba injected into the sheep, rabbit and fish had been feeding on *P. maltophilia,* and as such, upon sonication, it was likely that bacterial contamination existed. To ensure that results were not perturbed due to the presence of anti-*Pseudomonas* antibodies, the serum was absorbed prior to conducting an ELISA. To do this, diluted sheep anti- *Paramoeba* sp. sera in PBS (1: 100) was absorbed with PBS-washed sonicated *P. maltophilia* cells (final concentrations of 10 mg protein ml^{-1} of sera) for 1.5 h at room temperature and gently mixed intermittently. Also, washed live *P. maltophilia* cells were diluted to a relative concentration of the McFarland standard number 2- 4 and added to the tubes that already contained serum and sonicated *P. maltophilia* and incubated at room temperature for another one hour. This was to absorb all anti-*Pseudomonas* antibodies.

The solution containing APA was centrifuged at 1000g for 10 minutes and the supernatant was collected for measuring its antibody level. Efficacy of absorption after each absorption was determined by ELISA (using several different dilutions of antigen and antibody).

Performing ELISA:

Soluble antigen was coated to plates ranging in concentration from 0.1 to 20 μg protein per ml solution in borate coating buffer. Coated plates were left for incubation at 4°C for 16-24 hours. After incubation antigen was flicked off and wells were blocked by 100 μl blocking solution (1% gelatin for 30 minutes at 25°C). Plates were washed 5 times with DWT.

The absorbed sera was added to wells and plates were incubated for 90 minutes. Plates were washed and the appropriate antibody conjugated with HRP was added and after 90 minutes incubation and after washing, ABTS was added and the assay continued according to the general method described previously.

In order to ascertain the reactivity of specific antibody to *Paramoeba*, in each plate absorbed sera and non-absorbed sera was used. Also in each assay one plate was coated with sonicated *Paramoeba* while another plate was coated with sonicated *P. maltophilia*. Absorbed serum in *P. maltophilia* coated plates did not show activity suggested the optimal absorption of the serum.

In the *Paramoeba* ELISA, the dilutions of antigen, sera and conjugate used were similar to *Vibrio* and *Streptococcus* ELISAs. Soluble antigen was used in a dilution of 1-10 μg ml^{-1}, sera were diluted 100-200 times and monoclonal antibody and conjugate usually diluted 10 and 500-1500 times respectively. Humoral response of sheep, rabbit, and rainbow trout immunised with sonicated *Paramoeba* sp. antigen in 50% v v^{-1} FCA is shown in Figure 2.6.5. Immunisation of animals against *Paramoeba* is discussed later in this thesis (section 5.2).

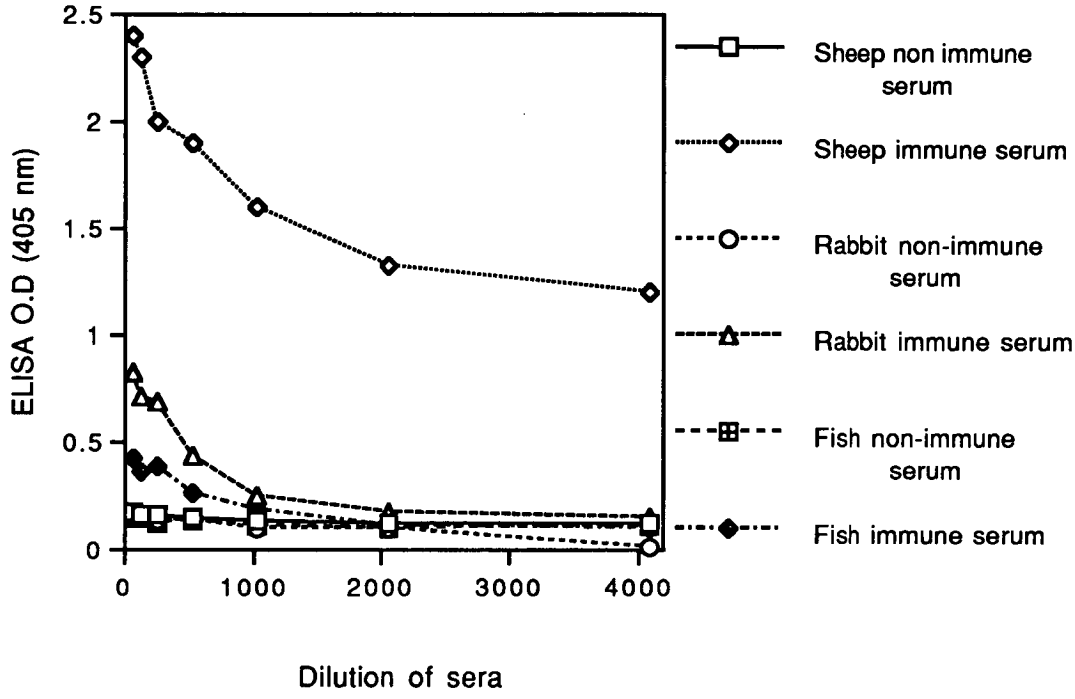


Figure 2.6.5: Optical density vs dilution (1: 2- 1: 4096) of sheep, rabbit and fish anti-*Paramoeba* sp.

2.6.6 Correction of ELISA's optical density (O.D) readings:

Unless otherwise stated all ELISA's optical densities were corrected by the method of plate correction factor (PCF) described by Whittington (1992). In each plate positive (high, medium and low positive) and negative controls were included as mentioned previously.

$$PCF=\frac{\text{Target O.D A/Actual O.D A + Target O.D B/Actual O.D B+ Target O.D C/Actual O.D C+TargetO.DD/A.O.D}}{4}$$

A, B, C and D respectively indicate ODs of highly positive, medium positive, low positive and negative; Target O.D, is mean O.D for each control over a period of time (at least 20 ELISA plates) and actual O.D is the mean of actual reading of duplicated wells.

The coefficient of variation for within daily test did not exceed more than 15% otherwise the test was repeated.

The ELISA positive-negative cut-off was defined as the mean O.D (405 nm) of negative sera plus 2 standard deviation. Sera with optical densities more than this reading were classified as seropositive.

2.7 Agar Gel immunodiffusion (AGID) test:

In order to observe antigen-antibody precipitation reaction between different sera and soluble antigens in the gel. Antigen (20 µl) with a concentration of 1 mg/ml was placed in centre well and immune bleed sera (20 µl each) of all three species were put in six peripheral wells (Figure 2.7).

The covered petri dish was incubated in a moist chamber overnight. The precipitation reaction in the gel was apparent between the immune sera and the antigen.

All sheep and rabbit antibodies against *V. anguillarum* and *Streptococcus* sp. showed strong precipitation reactions. However, precipitation of fish antibodies was not observed suggesting a lack of fish precipitin antibodies.

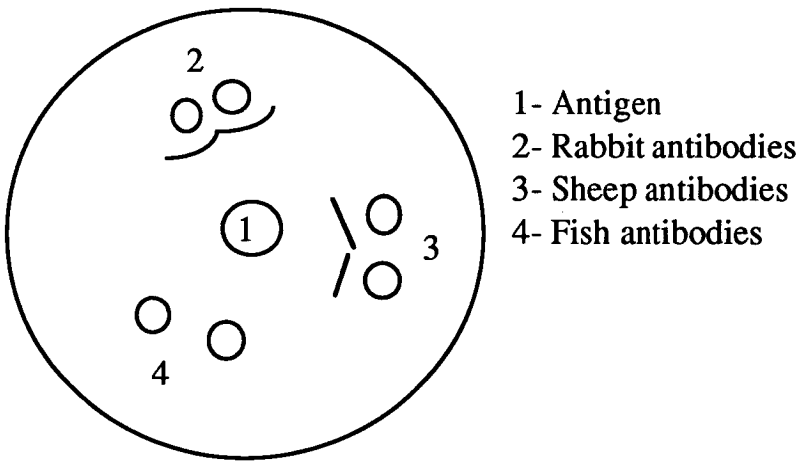


Figure 2.7: Configuration of agar gel immunodiffusion test

2.8 Slide agglutination test:

In order to observe agglutination reactions between antigen-antibody, this test was performed. Firstly sera were serially diluted up to dilution of 1: 200. Then one drop of serum from each dilution was put on the slide and a drop of antigen homogenised in PBS was added. The slide was rotated gently for 2-3 minutes and closely watched for evidence of an agglutination reaction.

The same procedure was conducted with 1: 20 dilution of antigen and bacterial suspensions from fresh colonies of bacteria (24 h. cultured) for optimising the test. By using low magnification with a stereo microscope agglutination could be seen at high dilutions.

Fish AVA showed higher agglutination up to dilution of 1024. This is while sheep and rabbit did not show agglutinating antibodies more than the titre of 1: 100 (Table 2.8.1).

Table 2.8.1: Agglutination titres of sheep, rabbit and fish AVA

Dilution of sera	Sheep	Sheep	Rabbit	Rabbit	Fish	Fish
	Aggl. titre	ELISA OD	Aggl. titre	ELISA OD	Aggl. titre	ELISA OD
1: 2	+++	2.312	+++	1.212	+++	1.441
1. 4	+++	2.451	+++	1.218	+++	1.504
1: 8	+++	2.514	+++	1.311	+++	1.512
1: 16	+++	2.511	+++	1.307	+++	1.451
1: 32	++	2.614	+++	1.318	+++	1.513
1: 64	+	2.741	++	1.510	+++	1.118
1: 128	-	2.762	+	1.618	+++	0.819
1: 256	-	2.851	-	1.567	+++	0.551
1: 512	-	2.231	-	1.101	+++	0.253
1: 1024	-	1.741	-	0.891	++	0.221
1: 2048	-	1.231	-	0.602	-	0.163
1: 4096	-	1.214	-	0.526	-	0.154

Aggl. titre= Agglutination titre

+++= highly positive ++= moderate positive += low positive -= negative

The results showed that ELISA is more sensitive than agglutination in detecting Ig against *V. anguillarum* in sera of sheep and rabbits but in the case of fish, agglutinating activity persisted to high titre. This result is explicable in terms of the nature of the Ig in the three sera. Fish with IgM would be expected to agglutinate much more efficiently than sheep/ rabbit where the Ig would have been mostly IgG.

Anti-*Streptococcus* sp. agglutination antibody titres were low in intraperitoneally immunised fish. However, agglutination titres in both sheep and rabbits' serum were detectable up to dilution of 1: 64. It is obvious that ELISA showed more sensitivity in three species detecting anti-streptococcal antibodies (Table 2.8.2).

Table 2.8.2: Agglutination titres of sheep, rabbit and fish ASA

Dilution of sera	Sheep Aggl. titre	Sheep ELISA OD	Rabbit Aggl. titre	Rabbit ELISA OD	Fish Aggl. titre	Fish ELISA OD
1: 2	+++	2.212	+++	0.534	++	0.341
1. 4	+++	2.201	+++	0.504	+	0.304
1: 8	+++	2.314	+++	0.518	-	0.212
1: 16	+++	2.310	+++	0.521	-	0.151
1: 32	+	2.310	++	0.517	-	0.113
1: 64	-	2.401	+	0.542	-	0.109
1: 128	-	2.512	-	0.621	-	0.110
1: 256	-	2.110	-	0.541	-	0.099
1: 512	-	2.612	-	0.482	-	0.111
1: 1024	-	1.402	-	0.392	-	0.087
1: 2048	-	1.321	-	0.311	-	0.092
1: 4096	-	1.210	-	0.272	-	0.095

Aggl titre= Agglutination titre

+++= highly positive ++= moderate positive += low positive -= negative

2.9 Fish husbandry

For all experiments fish were taken from the stock tank and acclimatised in temperature controlled (15°C) 4000 L fibreglass tanks (water flow of 25 L min^{-1}). Each tank had its own biofilter and temperature control system (Plate 2.9). Fish were fed (1.0% their body weight) commercial trout pellets (Gibson) twice daily . The protein and lipid contents of the diet were 45 and 17.5% respectively.

Water exchanges of 20-30% were conducted daily and tanks were cleaned every week (Table 2.9.1). All water added were dechlorinated with sodium thiosulphate when the water appeared to contain chlorine. Water quality parameters (pH, ammonia and nitrite) were measured and recorded usually prior to water exchange. For all experiments two temperature controlled tanks were used (for two replicates, each replicate for a treatment consisted of 10-12 fish). Fish were all identified individually using plastic T-bar anchor tags (Hallprint, Pty. Ltd, South Australia). The efficacy of these tags on fish was 98%. It was noted from different experiments that not more than 2% of tagged fish lost their tags up to 3 months post-tagging.

Table 2.9: Everyday husbandry of fish

Feeding*	at 9.00 a.m
Ammonia and nitrite test (if required)	9.30 a.m
Water changes (20-30%)	10.00 a.m
Cleaning and scrubbing of tanks (if required; usually twice a week)	10.00 a.m
Feeding (2nd time)	3.30 p.m
Final check of electricity, pumps and etc.	4.30 p.m

* 1% body weight

In those experiments for determination of the rate of clearance of antisera from fish serum, fish were kept in tanks and at particular intervals, the required number of fish were taken out. After blood collection, fish were returned to their tanks or euthanised.

Plate 2.9.1: Temperature controlled tanks with their own biofilters

In those experiments for determination of protection, fish were immunised in replicate groups and maintained in two tanks. Two days before the time of each challenge the required number of fish were taken out and transferred to an isolation unit "disease unit".

The disease unit consisted of two series of tanks (each tank 200 L), each series with its own biofilter system outside the unit (Plate 3). Biofilters outside of the unit

were insulated in order to keep the temperature constant (water flow of 15 L min^{-1} in each series of tanks). Every day the tanks were cleaned twice and the water change did not exceeded 20-30%. Fish in the disease unit were fed with commercial trout pellets twice daily. They were observed to 14 days post-challenge for disease signs and moribund fish were euthanised and collected for bacteriological tests. Surviving fish also were euthanised and sampled at the end of each experiment for isolation of pathogens.

Plate 2.9.2: Isolation (disease) unit (temperature controlled)

For Atlantic salmon used in the *Paramoeba* protection trial, two temperature controlled tanks inside the trout room were used. At the time of challenge of immunised fish, diseased fish from SALTAS fish farm were introduced into these tank and fish were watched for behavioural responses and samples (blood collection and direct gill samples) were collected. Gills were collected finally for confirmation of paramoebiasis after euthanising the fish.

2.10 LD_{70} determination

This section describes the materials and methods employed for LD_{70} determination trials of *V. anguillarum* and *Streptococcus* sp. including validation of the trials. The section applies to all protection experiments later in the thesis.

2.10.1 LD_{70} determination for *V. anguillarum*

Freeze dried *V. anguillarum* which was previously characterised and used in the production of anti-*V. anguillarum* antisera, was used as the challenge strain in all protection experiments for vibriosis. All challenge trials including LD_{70} determination and experimental challenge for determination of the rate of protection were conducted in freshwater.

2.10.1.1 Reconstitution and culture of *V. anguillarum*

The bacteria were reconstituted in nutrient broth, plated on blood agar or subcultured into tryptose soy broth (TSB) after two days incubation at 25°C.

2.10.1.2 Preparation of inoculum for LD_{70} determination

V. anguillarum was passaged four times through rainbow trout before use in the challenge experiment. Each time 5 fish (mean weight of 85 g) were anaesthetised and 0.1 ml of the bacterial inoculum (each fish with definite number of bacteria) was injected i.p into each fish. The kidney of several moribund fish was sampled and cultured on blood agar (BA). *V. anguillarum* was isolated after 48 h and subsequently injected to another group of five fish as mentioned above. The results of the enhancement of pathogenicity (disease signs in the inoculated fish) following multiple passages of the bacteria on fish are shown in Table 2.10.1.

Table 2.10.1: Induction of V. *anguillarum* pathogenicity following multiple passages on rainbow trout.

Passage through Normal Rainbow trout	LD_{70} value cfu* cells/ 0.1 ml	d.c** cells/ 0.1ml
1	6.1×10^7	3.53×10^8
2	5.2×10^6	3.48×10^7
3	4.3×10^6	2.53×10^7
4	1.0×10^6	1.1×10^7

* Colony forming unit ** Direct microscopic count

Table 2.10.1 demonstrates that the virulence of *V.anguillarum* for rainbow trout was enhanced nearly ten fold after four passages through the susceptible host. The results of this study were consistent with those of Song *et al.* (1980) who stated that the *V. anguillarum* infection level which killed at least 60% of control fish was not less than 10^7 cells ml^{-1}.

2.10.1.3 Calibration of the absorbance standard curve against viable bacterial count and direct microscopic count

In order to determine the definite number of passaged *V. anguillarum* with an LD_{70}, a two-day subculture on blood agar of passaged *V. anguillarum* was homogenised in PBS, pH 7.2 and the number of bacteria was adjusted to an expected number (to achieve LD_{70}) from $5x10^6$-$6x10^7$ ml^{-1} in ten dilutions by direct count and the density of bacterial cells were determined spectrophotometrically at 550 nm using an spectrophotometer. Ten groups of rainbow trout each group consisted of 10 fish were inoculated i.p. with each dilution. Also, a serial 10-fold dilutions of each inoculum (each of the dilutions mentioned above) was prepared in PBS and cultured (0.1 ml) on BA using a modified Miles and Misra (1938) method.

Figure 2.10.1.1 shows per cent mortality of fish with different direct count, absorbance and number of colony forming unit obtained during the course of optimising the LD_{70} for *V. anguillarum*..

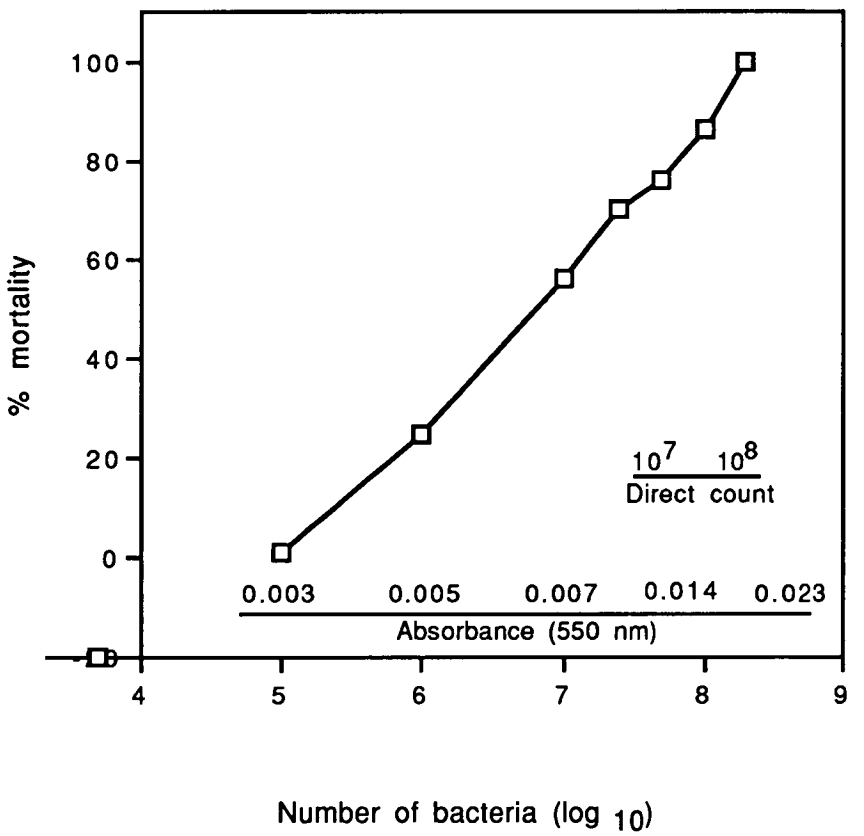


Figure 2.10. 1.1: Per cent of mortality vs number of bacteria [(cfu) (log_{10})], corresponding absorbance and direct count

The challenge was conducted so that a minimum of 70% of fish (unvaccinated) died of vibriosis. General linear analysis was used to fit responses with the proportion of fish that died as the dependent variable and log of dose (number of bacteria injected

i.p.) as the independent variable (Figure 2.10.1.2). A probit analysis computer package developed by A. Woods (University of N.S.W, Sydney) was employed to estimate the LD_{70} and its confidence limits. The obtained formula (Y= -3.081+ 5.194 X) was used to plot a straight line on the graph with Y on the vertical axis and X (log of dose) on the horizontal axis (Salsburg, 1986).

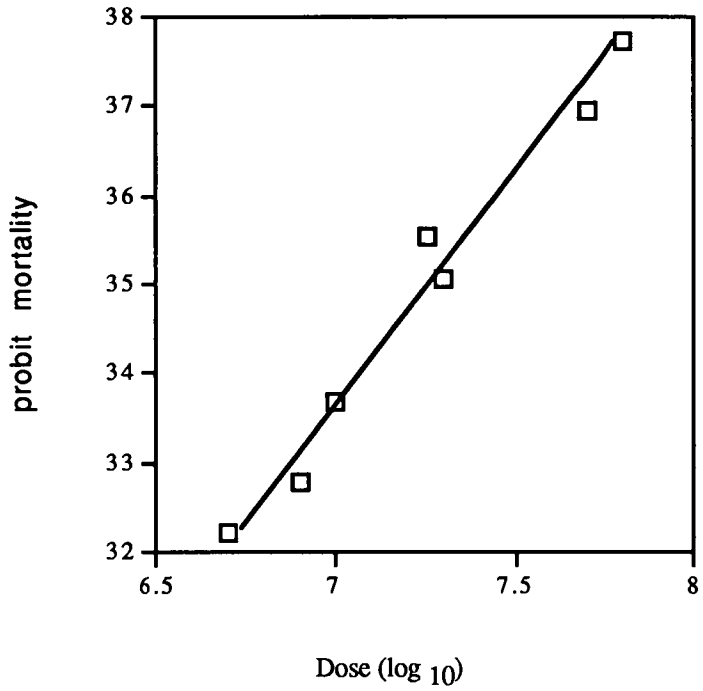


Figure 2.10.1.2: Dosage-mortality and confidence limits for *Vibrio anguillarum*

Therefore, an optical density of 0.016- 0.018 (550 nm) corresponding to approximately 1x 10^7- 4x 10^7 colonies forming units (cfu) 0.1 ml^{-1} was determined as LD_{70} value for *V. anguillarum.*

2.10.1.4 Preparation of *V. anguillarum* inoculum to achieve LD_{70} for experimental challenges:

For challenge of experimental fish, viable cells were taken from BA (passaged bacteria in fish and reisolated on BA), resuspended in PBS and transferred to a glass tube containing 5 ml sterile saline. The suspension was homogenised by vigorous agitation with a vortex mixer for 1 min to break up any bacterial clumps. Absorbance of cultures prior to challenge were determined spectrophotometrically as mentioned before and cell suspension was then adjusted to a turbidity of O.D of 0.016- 0.018 at 550 nm. *Vibrio anguillarum* cells were suspended to achieve a concentration of about 10^7 cfu ml^{-1}.

Viable count was carried out from the inoculum as described previously. This was to ensure that the number of bacteria injected into fish was in the desired range. A direct microscopic count was also carried out using a Helber counting chamber.

However, in each preparation of the desired number of cfu, an approximate number of bacteria was obtained e.g. 2x 10^7 or 4x 10^7 etc instead of exact number of bacteria due to time to time differences.

After each challenge, several sterile kidney samples were collected from diseased fish in aliquots and frozen for the use in the next challenge. In the next challenge, the frozen samples were thawed and passaged i.p. into 5 naive fish. *V. anguillarum* was reisolated from kidney samples and the number of bacteria was adjusted to the known absorbance and direct count. This bacterial suspension was used as inoculum in the experimental challenge.

2.10.1.5 Experiments to determine the relative per cent survival (RPS):

In the first challenge test, all the experimental fish, as well as the unvaccinated controls were injected i.p. with 0.1 ml of a dilution of live, virulent bacteria, which corresponded to 2x 10^7 cfu per fish. Therefore, the mortality rate between trials had a range (70- 85%). The challenge was accomplished using a single i.p injection of viable cells in PBS. Challenged fish were observed for 14 days and dead fish were collected, identified and counted daily. Mortalities were examined grossly for visible lesions and examined microscopically by the use of fresh tissue preparation. Tissue impression smears were stained by Gram's method. Kidney samples of recent mortalities were examined by culture on BA (containing 1.5% NaCl at 25°C for 2 days) and agglutination test of isolated colonies with morphology consistent with control *V. anguillarum* cultures was carried out with rabbit anti-*V. anguillarum* to verify that the *V. anguillarum* was responsible for the death. Mortalities were attributed to *V. anguillarum* only when the organism was isolated in pure culture from the dead fish.

Diseased fish were those that showed common clinical signs as follows; swam slowly and ignored food, laboured swimming, postural disturbances, tachybranchia, abdominal distension, congestion of the fin bases and vent, mucoid yellow faecal casts and marked cutaneous blackening. Of the fish that died of vibriosis, gross lesions were observed in about 50% of the mortalities. The most consistent finding was blackening, reddening of vent and presence of excessive volumes of peritoneal fluid (Plate 2.10.1).

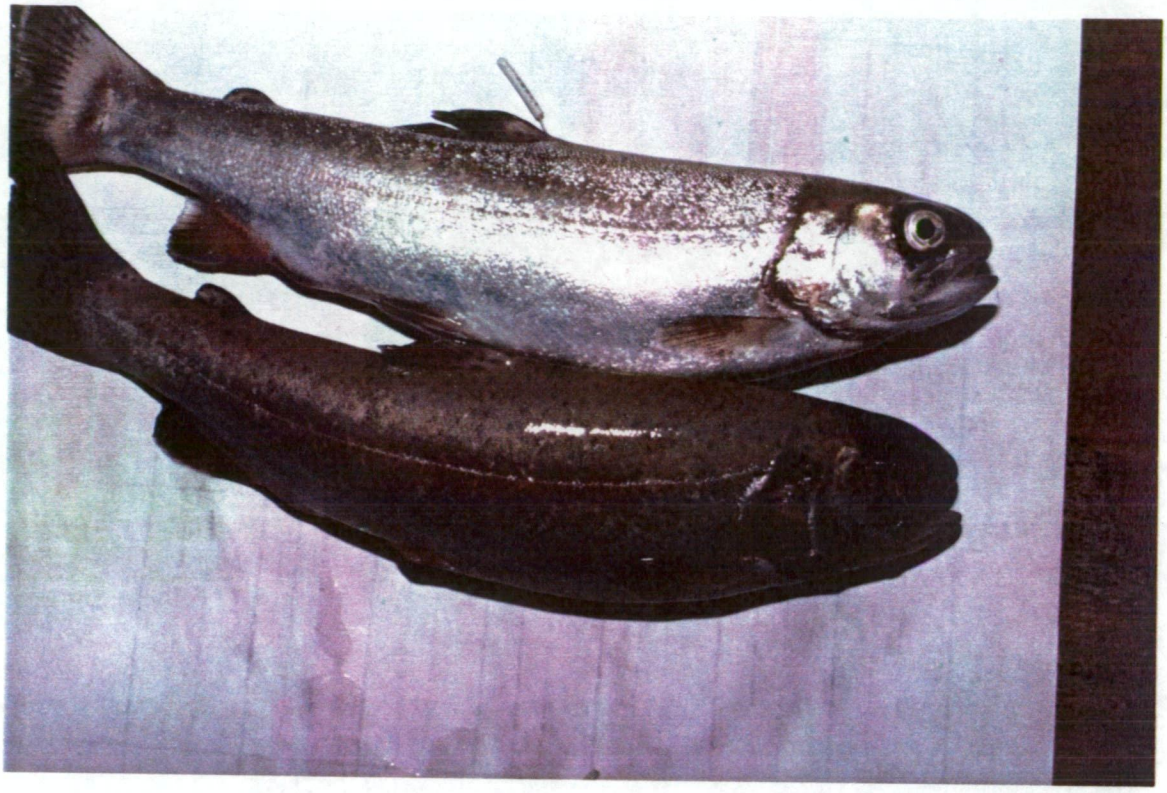


Plate 2.10.1: Freshly dead fish due to peracute vibriosis after i.p. injection with virulent *V. anguillarum* (LD_{70}) showing haemorrhages in the base of fins, red and swollen vent

Two weeks after the commencement of each challenge test, the surviving fish were counted and euthanised and their kidney materials were cultured on BA plates in order to monitor the carrier status of the fish.

Relative per cent survival was determined by the percentage of mortality. Immunity against V. *anguillarum* in actively and passively immunised animals was expressed in terms of relative per cent survival (RPS) as described by Amend (1981).

$$RPS = \left[1 - \frac{\%\ \text{Mortality in vaccinated}}{\%\ \text{Mortality in control}}\right] \times 100$$

2.10.2 LD_{70} determination for streptococcosis

The *Streptococcus* sp. isolated in 1987 (the same strain used for raising antisera) was passaged six times through rainbow trout (mean weight of 75 g) to enhance its virulence and an LD_{70} was determined as described for LD_{70} determination for *V. anguillarum.* with some differences as follows:

2.10.2.1 Effect of passage on pathogenicity of *Streptococcus* sp.

During the passage procedure it was noticed that the injected dose of *Streptococcus* sp. decreased markedly from 2000 to 5 cells. This demonstrates that the virulence of *Streptococcus sp.* for rainbow trout was enhanced significantly through the susceptible host. The results of pathogenicity increment through healthy rainbow trout are shown in Table 2.10.2.

Table 2.10.2: Induction of *Streptococcus* sp. pathogenicity following multiple passage on rainbow trout

Passage	LD_{70} value cfu cells/0.1 ml
Normal Rainbow trout	
1	2000-3000
2	2000
3	1000
4	250-350
5	30-40
6*	3-5

*Dilution of 10^{-6} of density of 0.099 (350 nm) of *Streptococcus* sp.

2.10.2.2 Calibration of absorbance standard curve against viable bacterial count

For i.p. inoculum, a two-day subculture on BA was homogenised in PBS. Bacterial suspension was adjusted to an optical density (at a wavelength of 350 nm). Since large numbers of bacteria were expected in the solution, it was necessary to make a wide range of dilutions in order to obtain a countable plate from this suspension. Therefore serial 10-fold dilutions (ten dilutions) in PBS, pH 7.2 was prepared from the suspension with a known optical density. Ten groups of fish (each group consisted of 10 fish) were injected with the 10 different dilutions of known bacterial density.

As streptococci form chains of cells, it is impossible to know the actual individual viable count. Therefore, it is more appropriate to consider the number as colony-forming units, rather than individual cells (Rasheed and Plumb, 1984). Per cent mortality of groups of fish challenged i.p. by different number of bacteria (3-100 cfu) is shown in Figure 2.10.2.1.

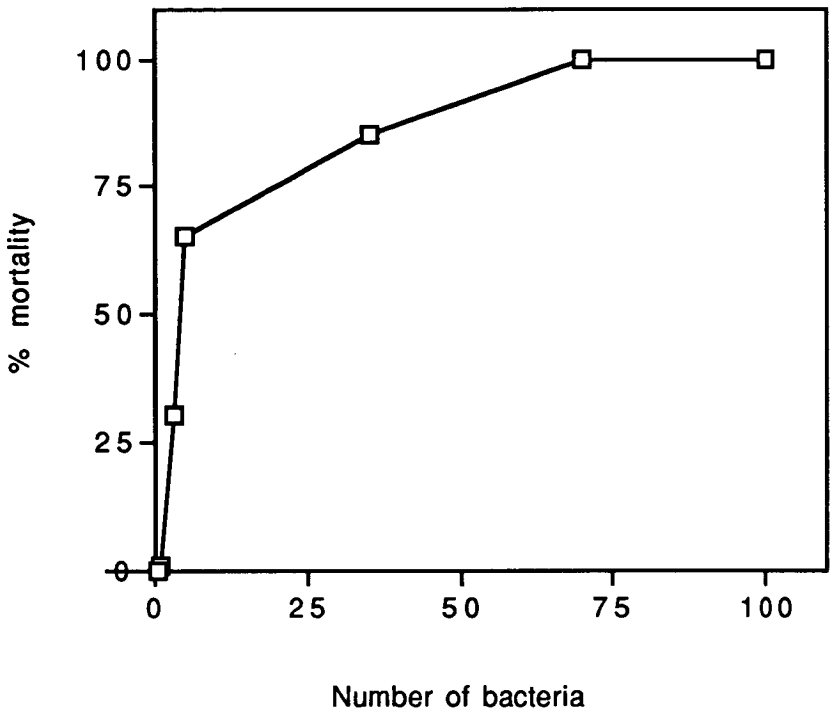


Figure 2.10. 2.1: Percent mortality due to streptococcosis vs number of bacteria (cfu)

Passaged bacteria (> 50) often gave high per cent mortality. Number of less than 50 the bacteria often resulted in mortalities of 70-90 per cent. Probit mortality for streptococcal 70% lethal dose was plotted using general linear analysis as described in previous section (Figure 2.10.2.2). The formula for calculation of Y was Y= -4.595 + 7.965 X as described previously.

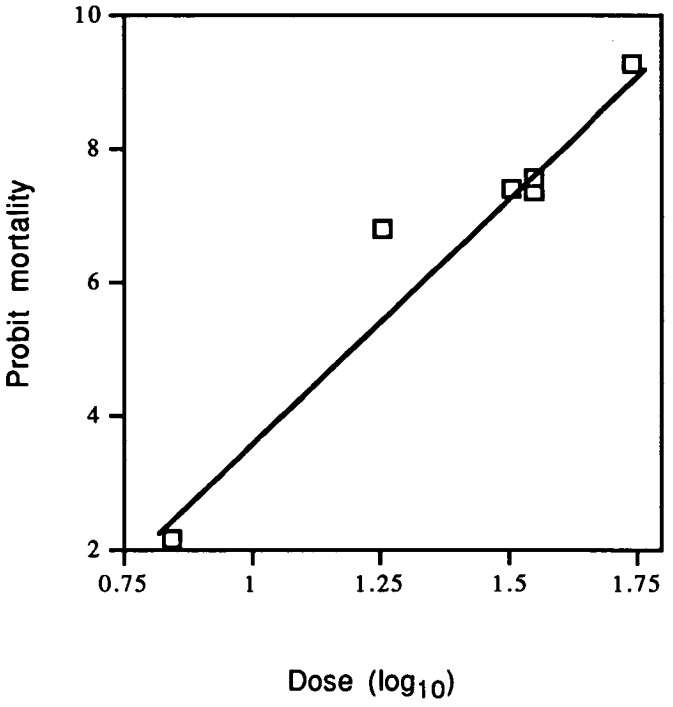


Figure 2.10.2.2: Dosage-mortality and confidence limits for *Streptococcus* sp.

2.10.2.3 Experiments to determine relative per cent of survival

Experimental fish were injected i.p. with 0.1 ml of various dilutions of bacteria. Inoculated fish were kept in the isolation unit in tanks supplied by freshwater at temperature of 18°C and the fish were watched for 14 days and dead fish were removed daily. In spite of the careful attention for achieving a desired number of bacteria for each challenge, a variation in the number of bacteria appeared that resulted in LD more than 70-75 per cent in most of the challenge trials.

Following inoculation, fish were observed twice daily for 14 days. Clinical signs often began after 72 h with mortalities subsequently which ceased 8 days later. Activity, response to feeding, development of lesions and mortalities were recorded. Dead or moribund fish were removed from tanks twice daily.

Any fish that died within the first day after challenge were not counted as having died of streptococcosis, as the trauma of injection was considered to be more likely responsible.

Freshly dead fish from each group were collected and their kidneys inoculated aseptically onto blood agar for the isolation of *Streptococcus* sp. Gross lesions were observed in about 95% of the mortalities. The most consistent finding was blackening and presence of exophthalmos (popeye) and severe eye haemorrhage outside and inside eyes. Streptococcal disease produces a typical septicemia and pronounced exophthalmos (popeye) often leading to complete degeneration of the eye (Plate 2.10.2). In acute cases fish become dark, swim sluggishly and cease feeding. Fungal invasion (saprolegniosis) was often observed on the skin of moribund fish All

moribund fish were examined by autopsy and kidney samples were inoculated on brain heart infusion agar (BHI). The bacteriological procedure used, consisted of streaking of kidney material on BHI and BA followed by identification of the isolates by means of colony appearance, haemolysis, Gram's stain and confirmation by rabbit anti- *Streptococcus* sp. antiserum by the slide agglutination test.

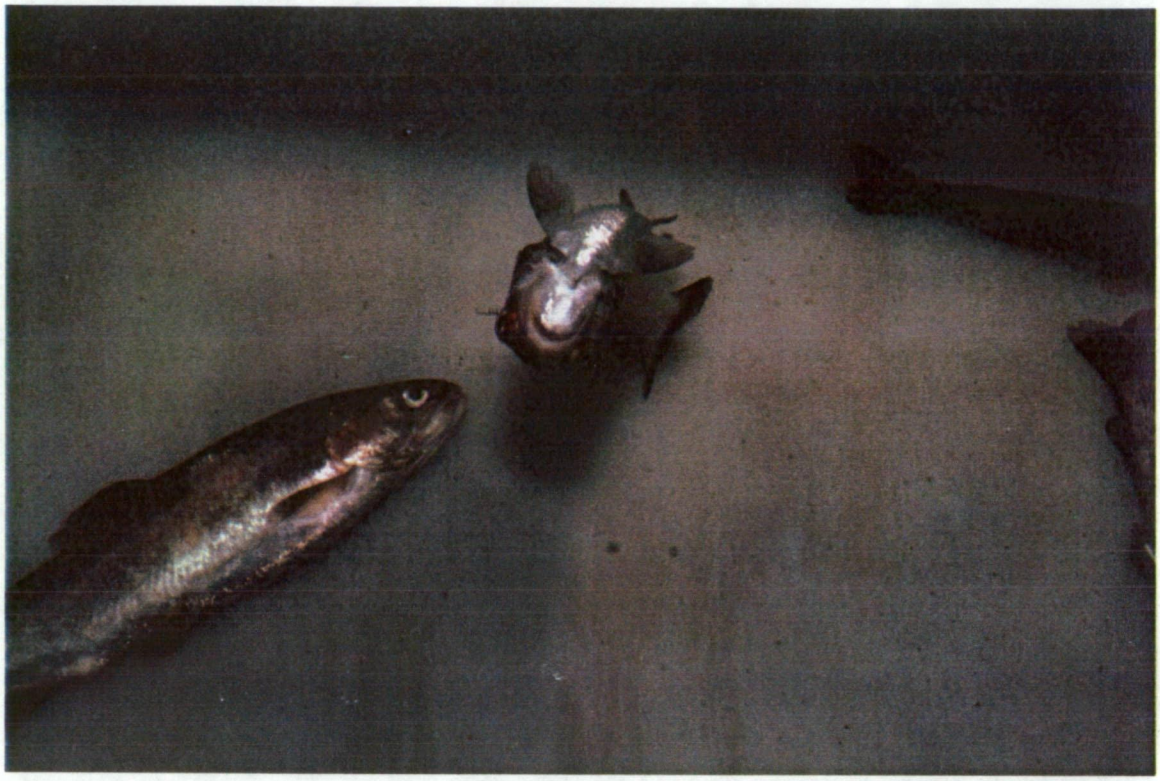


Plate 2.10.2: Freshly dead fish due to streptococcosis after i.p. injection of virulent *Streptococcus* sp. (LD_{70}), showing typical exophthalmia and haemorrhages in eyes.

As it is documented (Boomker *et al.,* 1979; Bragg and Broere, 1986; Carson, 1990; Munday *et al.,* 1993) there was a very obvious relationship between water temperature and the occurrence of clinical streptococcosis in rainbow trout. The importance of water temperature in precipitating outbreaks of streptococcosis of rainbow trout was obvious in these experiments.

In real terms, the results indicated that very few bacteria were required to kill fish. The pathogenicity of the *Streptococcus* sp. with regard to it's low lethal dose concentration may be attributed to the fact that Gram-positive bacteria produce exotoxins which can be highly toxic at very low concentration. In gulf killifish, the LD_{50} of a non-haemolytic group B *Streptococcus* sp. was 1.4×10^4 cells at 96 h and 7.5 x 10 cells at 168 h.

It is for the first time that the LD_{70} of the *Streptococcus* sp. cells used for these experiments is reported as low as 5 cfu.

Relative per cent survival for resisted fish in challenge was calculated as described for *V. anguillarum* challenge. After each challenge, several kidney tissues from freshly dead fish due to streptococcosis were frozen as the source of bacteria for the next challenge as described in previous section.

2.11 Horizontal transmission of vibriosis and streptococcosis

This section describes method of horizontal transmission of vibriosis and streptococcosis in freshwater. The method and the results obtained are described here which applies to all protection experiments later in the thesis.

2.11.1 Horizontal transmission of vibriosis, natural transmission (cohabitation) of fish with the diseased fish:

In order to evaluate what percentage of mortalities was due to the horizontal transmission of *V. anguillarum*, 20 rainbow trout (non-immunised) in two replicates were cohabited with the i.p. challenged fish (LD_{75}).

Results of the cohabitation of fish with the diseased fish showed 20% mortality due to vibriosis (positive results from kidney culture of all mortalities). Mortality of these fish happened at 6-8 days post-cohabitation which was longer than the 3 days incubation period for those fish which were challenged by i.p. route. The remaining survivors of this group were negative in culture 14 days post-challenge.

Results of this experiment showed 20% mortality of the cohabited fish and the organism was not isolated from the kidney of survivors .The results from the cohabitation of fish suggest that the disease can be transmitted horizontally through freshwater via contaminated excretion of fish or feeding fish with contaminated materials. Although, vibriosis is usually regarded as a marine disease, it is pertinent that outbreaks have been reported in freshwater (Ross et al., 1968; Hacking and Budd, 1971; Giorgetti and Ceschia, 1982).

2.11.2 Horizontal transmission of streptococcosis

In order to evaluate the horizontal transmission potential of *Streptococcus* sp. in the contaminated water, 20 non-immune fish were cohabited with the i.p. injected fish with *Streptococcus* sp (LD_{85}) in freshwater. The results showed 40% mortality in non-immune fish. Mortalities happened 7-8 days post-challenge and the bacteria were isolated from both kidney and brain. Survivors were all negative in culture after 14 days after challenge. This shows that the pathogen spreads horizontally via the water column.

2.12 Cumulative mortality of experimental fish in challenge

2.12.1 Cumulative mortality of experimental fish in challenge with *V. anguillarum*

The pattern of mortality of fish after i.p. challenge with the virulent *V. anguillarum* in all experiments determining LD_{70} and those evaluating the protection rates of immunised fish (protection experiments described later) which applies to all protection trials for vibriosis is shown in Figure 2.12.1.

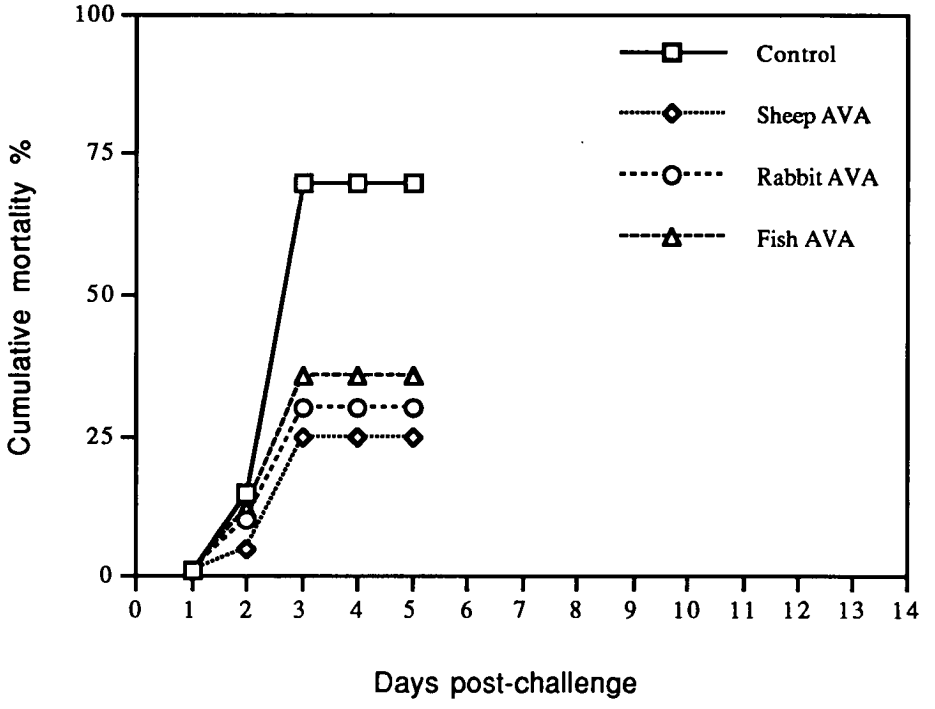


Figure 2.12.1: Cumulative mortality of experimental fish in challenge experiments with *V. anguillarum*

It was noted that the fish either die up to 5 days post-challenge (mainly non-immune fish) or survive the challenge (mainly immune fish).

2.12.2 Cumulative mortality of experimental fish in challenge with *Streptococcus* sp.

The pattern of mortality of fish after i.p. challenge with the virulent *Streptococcus* sp. in all experiments determining LD_{70} and evaluating the protection rates of immunised fish (protection experiments described later) which applies to all protection trials for streptococcosis is shown in Figure 2.12.2.

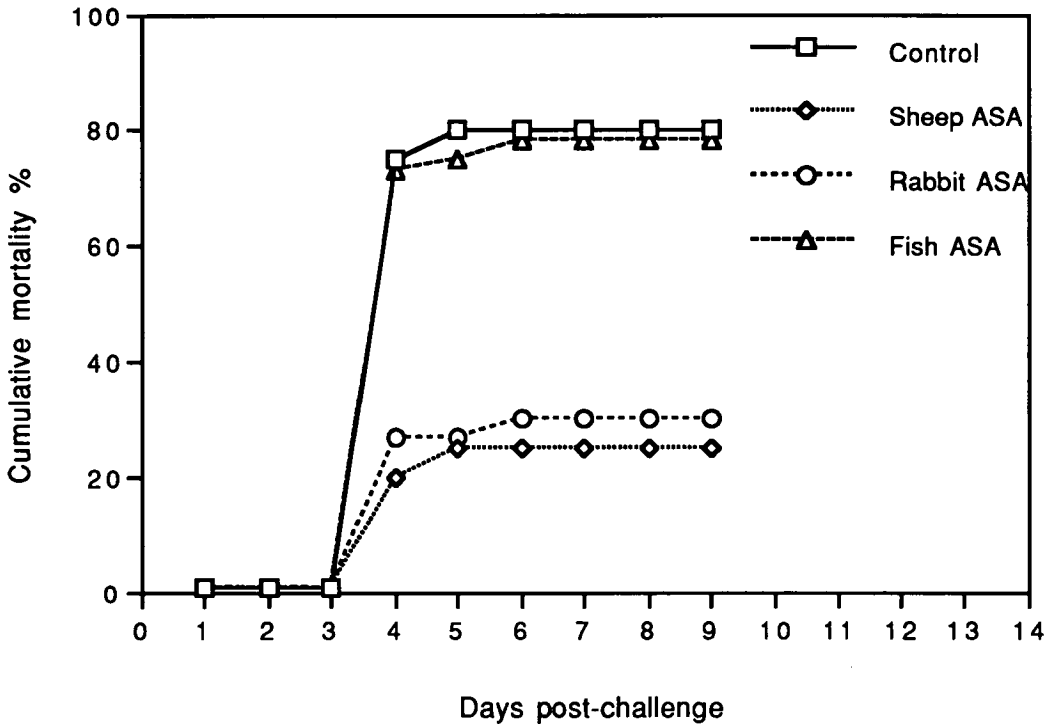


Figure 2.12.2: Cumulative mortality of experimental fish in challenge with *Streptococcus* sp.

2.13 **Statistical analysis:**

ELISA O.D: A paired t-test was employed to compare two observations (mean ELISA O.D) within a group of fish. Comparisons between more than two groups of fish (mean ELISA O.D) in a treatment and between treatments in an experiment, were carried out using one way analysis of variance (ANOVA) (JMP software package for Macintosh computer). Prior to performing ANOVA, homogeneity of variance was determined using Cohran's test. If variance were heterogeneous, the data were transformed, using either square root and log(x+1). Unless otherwise stated, a probability level of less than 0.05% was considered significant.

Relative per cent survival (RPS): At the end of each challenge trial, mean RPS was calculated from RPSs of replicates and showed with its S.E (±S.E.). The percentage mortality in each group was compared statistically with the percentage mortality of other groups within an experiment using a chi-squared test and pairwise comparisons were done with the Mann-Whitney Rank Sum Tests.

CHAPTER 3.0

VIBRIOSIS, PASSIVE AND ACTIVE IMMUNISATION AND EVALUATION OF PROTECTION

3.1 Introduction /literature review:

Vibriosis is an infectious disease of many marine and estuarine fishes of the world. The disease is often the major problem in rearing salmonid fish in seawater, causing considerable economic losses in the marine fish farming industry particularly infections with *V. anguilarum* (Austin and Austin, 1993). The severity of vibriosis has increased proportionately with the development and expansion of fish farming worldwide. In severe cases of vibriosis there may be up to 40 per cent mortality. Recent figures suggest that in Japan alone annual losses due to the disease exceed 11 million pound sterling (Smith, 1988).

3.1.1 *Vibrio anguillarum* infection:

The causal agent of 'red-pest' in European eels (*Anguilla anguilla*) was first isolated by Canestrini (1893) who described the organism as *Bacterium anguillarum.* In 1907 further cases among eels in Sweden were investigated by Bergman (1909) who coined the name of *Vibrio anguillarum.* For many years all pathogenic vibrios were ascribed to this taxon (Austin and Austin, 1993).

Vibriosis as a bacterial disease of salt-water and estuarine fish has gained considerable notoriety in mariculture, where it has become a major limiting factor in the successful rearing of salmonids (Mahnken, 1975). Evidence is also accumulating that the disease may occur in freshwater (Muroga, 1975; Ohnishi and Muroga, 1976; Ghittino and Andruetto, 1977; Giorgetti and Ceschia, 1982). This suggests that vibriosis is an extremely widespread problem.

However, it would appear that in particular geographical areas and culture systems, vibriosis is in fact, a syndrome caused by a multiplicity of *Vibrio* species such as *V. anguillarum* (*Listonella anguillarum;* proposed by MacDonell and Colwell, 1985), *V. alginolyticus, V. carchariae, V. cholerae* (non-01),*V. damsela* [(*Listonella damsela* proposed by MacDonell and Colwell, 1985) and reclassified as *Photobacterium damsela* (Smith *et al,* 1991)], *V. fischeri, V. harveyi, V. ordalii, V. salmonicida, V. splendidus,* and *V. vulnificus* biogroup 2 (Austin and Austin, 1993).

V. anguillarum itself and the two species subsequently designated *V. ordalii* (Schiewe *et al.* 1981) and *V. salmonicida* (Egidius *et al.* 1986) appear to be the most pathogenic. *V. ordalii* and *V. salmonicida* are principally associated with disease in Pacific and Atlantic salmon respectively (Inglis *et al,* 1993).

This discussion will be restricted to vibriosis caused by *V. anguillarum.* However, confusion has resulted from the multiplicity of names used to describe the disease. Some workers use 'salt-water furunculosis' (Rucker, 1963), 'boil-disease'

(Kubota and Takakuwa, 1963) and "ulcer disease" (Bagge and Bagge, 1956) as well as the universally accepted name of 'vibriosis'.

3.1.2 **Characterisation of the pathogen**:

The taxonomy of *V. anguillarum* has had a long history. The complexity in the taxonomic understanding of this organism began with the recognition by Nybelin (1935) of two biotypes which where differentiated on the basis of biochemical properties; there was some serological cross-reactivity between species. A further group, i.e biotype C was recognised by Smith (1961) and two further biotypes, i.e D and E, were later described. However, in view of modern thought and approaches on bacterial taxonomy these descriptions are inadequate (Austin and Austin, 1993).

Type C deserves special mention because this was proposed for Japanese strains derived from rainbow trout, *V. piscium* var. *japonicus* (Hoshina, 1956). Researchers who were involved in characterisation of *Vibrio* species isolated from fish had found a necessity to rectify the taxonomic chaos surrounding the fish pathogenic vibrios. Håstein and Smith (1977) and Bryant *et al.* (1986) established a numerical classification of species of *Vibrio* and related genera. These researchers examined 1091 strains of the Family Vibrionaceae with a set of 142 characters. Nowadays, many laboratories which are involved in the isolation of fish pathogens use these guidelines to characterise isolates.

V. anguillarum serotype C (01) is the only type which has been isolated from rainbow trout and Atlantic salmon in Tasmania. The serovars of *V. anguillarum* isolated from other locations in Australia are not known but serotype B (03) has been isolated in New Zealand (Munday, 1988; Carson, 1990). *V. anguillarum* serotype C (01) isolated from Tasmania was used in this research project.

3.1.3 **Epidemiology**:

V. anguillarum, the causative agent of the disease has been isolated from salmonids and mullet in Australia (Carson, 1990). Isolation of *V. anguillarum* and other fish pathogenic *Vibrio* sp. from farmed salmon in Tasmania indicates the distribution of these *Vibrio* species along the entire eastern seaboard in habitats where water temperatures range from 9-31°C and salinities between 1-35‰ (Cameron *et al.*, 1988). Vibriosis has a global distribution with epizootics on all continents and in a wide range of fish species.

3.1.4 **Pathogenicity mechanisms**:

V. anguillarum constitutes part of the normal microflora of the aquatic environment and may constitute part of the natural microflora of marine fish (West and Lee, 1982; Muroga *et al.*, 1986). There are uncertainties as to how the organism transforms from an apparent commensal to a pathogen. Infection through adhesion of the organism to the skin, colonisation, growth and penetration of skin then

subcutaneous tissue followed by spreading all over the body, seems to be one possible mode of infection (Funahashi *et al.* 1974). Penetration is probably accomplished by the bacteria using their lytic enzymes or by inducing the host to phagocytosis them (Evelyn, 1984).

Ransom (1978) postulated that infection probably begins with colonisation of the posterior gastro-intestinal tract and rectum. This has been demonstrated experimentally with mortalities occurring when an inoculum was introduced via the anus to avoid exposing the pathogen to the secretions of the stomach (Chart and Munn, 1980). However, under natural conditions it is an unlikely route of entry. Evelyn (1984) concluded that the gill is a likely tissue for the entry of pathogenic bacteria into fish. It provides the fish with only a single layer of fragile cells as a barrier between the circulatory system and the external environment. Indeed, large particles, including killed *V. anguillarum* cells, appear to gain entry into fish via gills, even without obvious traumatisation of the site (Smith, 1982; Tatner and Horne, 1983).

Two main factors are associated with outbreaks of vibriosis due to *V. anguilarum.* The first and most important one is stress, such as acclimatisation to seawater and overcrowding (Snieszko, 1974). The other important factor is water temperature when it rises over 10°C. Rapid changes in water temperature, poor water quality; high amounts of organic material (faeces, etc.), low oxygen level and high suspended solids may provide an opportunity for this bacterium to build-up and may be involved in the occurrence of the disease. Skin lesions due to trauma or poor water quality may also provide a suitable portal of entry for the bacteria (Snieszko, 1974).

A detailed study of the pathogenesis of vibriosis in young coho salmon was undertaken by Harbell *et al.* (1979) who induced acute vibriosis by bath exposure to *V. anguillarum* and noted anaemia and leucopenia. They also obtained evidence for widespread cell and tissue destruction and loss of osmotic control. However, injection of fish with *V. anguillarum* endotoxin, culture supernatant and cell lysates, singly or in combination did not reproduce these clinical signs.

The question of how *V. anguillarum* manages to proliferate in the host and the nature of the virulence mechanism has been largely answered by the work of Crosa, (1980); (Crosa and Hodges, 1981). Virulent strains of *V. anguillarum* have an efficient iron-sequestering system (siderophores) that permits growth under conditions of iron limitation found in the host. In some strains of *V. anguillarum*, the iron-sequestering system is chromosome-determined (Toranzo *et al.*, 1983). However, a correlation between the presence of a 50 megadalton plasmid and the virulence of *V. anguillarum* was found (Crosa *et al.*, 1977). Subsequent experiments by Crosa (1980) and Crosa and Hodges (1981) demonstrated that the plasmid enhanced virulence by coding for iron chelation, which allowed *V. anguillarum* to successfully compete with the available iron in the fish tissues (Crosa, 1980).

Trust *et al.* (1981) proposed that the virulence of fish pathogenic vibrios may also be related to bacterial adhesion to target cells on host gill and gut epithelia. They found that neither adhesive nor haemagglutination functions were plasmid-coded. *In vitro* work has shown that *V. anguillarum* cells preferentially adhere to excised sections of mid and upper fish gut in preference to lower gut regions (Horne and Baxendale, 1983). Munn (1978, 1980) demonstrated haemolysin as a further virulence factor.

More recently, proteases of 36 kDa have been implicated with virulence (Inamura *et al.*, 1984; Kodama *et al.*, 1984). Finally, a zinc metalloprotease has been associated with invasive processes (Norquist *et al.*, 1990).

3.1.5 **Clinical signs and pathology**:

Vibriosis can occur in forms ranging from peracute to chronic. Peracute vibriosis is indicated by no defined clinical signs except occasional periorbital or abdominal oedema (Inglis *et al.*, 1993). In the acute stage fish have no clinical signs (Baudin Laurencin and Egidius, 1985), or dark skin swellings which ulcerate to release necrotic tissue containing very large numbers of bacteria (Inglis *et al*, 1993). Clinical signs include inappetence, lethargic behaviour and melanisation (Carson, 1990) and the presence of large haemorrhagic lesions (Munday, 1988). As the disease progresses, fish may develop hyperaemic prolapsed vents, visible congestion at the base of the fins and in chronic cases swollen haemorrhagic lesions are usually located deep in the muscle layers on the flanks of the fish (Carson, 1990).

Internally, there may be splenomegaly in acute cases (Baudin-Laurencin and Egidius, 1985) and more often skin lesions, fin erosion, haemorrhages in skin, muscles, and in all internal organs, splenic enlargement and liquefaction. The gut and rectum may be distended and filled with clear viscous fluid (Austin and Austin, 1993). Chronically infected fish generally have large granulating lesions deep in the muscle, pale gills and fibrinous adhesions between viscera and parietal peritoneum (Inglis *et al.*, 1993).

Histological findings in peracute cases include cardiac myopathy, renal and splenic necrosis associated particularly with the erythroid haemopoietic tissue and periorbital oedema (Inglis *et al.*, 1993). The bacterial cells appear to be uniformly distributed throughout the affected tissues, although the greatest concentration is in the blood (Tajima *et al.*, 1981). None of these lesions are specific for vibriosis and the diagnosis must be established by bacteriological techniques (Baudin Laurencin and Egidius, 1985).

3.1.6. **Isolation of the pathogen**:

Vibrio species are curved to straight, motile by one or more polar flagella, Gram negative oxidase positive rod-shaped bacteria (Carson, 1990). The pathogen may be isolated from the infected organs by using tryptic soy agar (Traxler and Li, 1972),

thiosulphate-citrate-bile sucrose agar (Bolinches *et al.*, 1988), nutrient agar (Muroga *et al*, 1976 a, b) and brain heart infusion agar (Tajima *et al.*, 1981), usually more profusely in the presence of sodium chloride at 0.5 to 3.5% (w v^{-1}) or seawater (35‰) with incubation at 15-25° C for periods of up to 7 days.

Identification can be made by biochemical tests, partial 16 S rRNA sequences, a specific 16 S rRNA oligonucleotide probe (Rehnstam *et al.*, 1989). The detection of a thermolabile O-antigen termed the k-1 antigen by slide agglutination (Tajima *et al.*, 1987) is an other diagnostic test.

3.1.7 **Treatment**:

Vibrio anguillarum may cause a primary disease in fish or it may be a secondary invader. In either case, treatment with antibacterial drugs is common and frequently contributes to resolution of the problem. Antibiotics are administered to fish either orally via medicated food, by bath or by injection.

In any antibacterial treatment protocol, drug residues in tissues, particularly muscle, should be considered. Also antibiotic resistance and incomplete therapy are frequent problems in fish medicine. In Japan, Aoki *et al.* (1974) found 65 of 68 random bacterial isolates from fish carried transferable drug resistant factors (R plasmid). In a further study, Aoki *et al.* (1981) identified such transferable R plasmids in 165 of 250 strains when they used combinations of six commonly used antibiotics. Antibiotic resistance problems from fish pathogens have also been investigated by Aoki (1988), Høle *et al.*, (1992), Hjeltnes *et al.*, (1987) and Inglis *et al.*, (1991).

Doses of oxytetracycline at 75 mg/kg body weight/day for 10 days requires a withholding period of 60 days at >10°C (Salata and Liestøl, 1983). McCracken *et al.* (1976) established that trimethoprim remained in rainbow trout muscle for 77 days after administration.

Appendix VII lists active doses of the most commonly antibiotics for treatment of vibriosis in aquaculture.

Anti-microbial agents were used as bath treatments or as food additives but drug resistance restricted their use. Every effort must be made to reduce stress on the fish such as: overcrowding, and warm water. Fish which are still feeding can be treated with antibiotics/ antibacterials in the food.

Medical treatment is expensive as well as hazardous to the ecosystem and, therefore, efforts have been made to find other means of protection against fish diseases, such as change in management, isolation and vaccination program (Harrell *et al.*, 1975; Antipa, 1976; Gould *et al.*, 1979). As well as changes in management, it might also be possible to improve resistance against vibriosis through selective breeding (Gjedrem and Aulstad, 1974).

3.1.8 **Disease control and prevention:**

Vibriosis control programs in salmonids frequently incorporate vaccination (Evelyn, 1984). Therefore, many attempts have been made to develop commercial vaccines for control of the disease. Vaccines against vibriosis are the most successful of any of the available vaccines against major diseases of fish (Smith, 1988).

3.1.8.1 **Immunisation**

Active immunisation is based upon the immune response (both antibody mediated immunity and cell mediated immunity) of the animal to the foreign molecules (antigens). Bacterial vaccines are preparations of antigens derived from pathogenic organisms. The first commercial product licence for a vaccine for fish was granted in the USA in 1976 for use against enteric redmouth (ERM) (Ellis, 1988a). Since then product licences have been granted for *Vibrio* vaccines in many parts of the world and vaccines against several fish diseases such as furunculosis, cold water vibriosis, and vibriosis have proved to be of great commercial value (Ellis, 1988a). Commercial vibriosis vaccines in the northern and southern hemispheres contain mixtures of the most commonly encountered local species, generally *V. anguillarum* and *V. ordalii*. Most of these vaccines consist of simple inactivated whole cells and extracellular products. Also in a few cases the bacteria have been lysed either physically or chemically. The immunogenicity of *V. anguillarum* in these vaccines appears to be dominated by heat-stable (to 100-121°C) lipopolysaccharides in the cell wall. These high molecular-weight compounds, 100 kDa (Evelyn and Ketcheson, 1980), are considered to confer protection to the recipient host. The lipopolysaccharides present in the cell walls of the fish pathogenic vibrios account not only for their serotypic specificity but apparently also for most or all of their immunogenicity (Johnsen, 1977; Aoki *et al.*, 1981). Sonicated heat-killed vaccines in adjuvant, also stimulate elevated levels of antibody in the skin and mucus (Harrel *et al.*, 1976; Evelyn, 1984). The protective antigen in *Vibrio* vaccines appears to be the lipopolysaccharide which is a T-independent antigen (Bly *et al.*, 1986a).

Chart and Trust (1984) isolated two minor proteins with molecular weights of 49-51 kDa from the outer membrane of *V. anguillarum* and these were potent antigens. A weakly antigenic protein, with a molecular weight of approximately 40 kDa was also present. Perhaps these antigens are heat-labile, protective, and explain the reasons for the greater protection achieved with formalin-inactivated vaccines compared to heat-killed products (Kusuda *et al.*, 1978; Itami and Kusuda, 1980). However, Gould *et al.* (1978) reported both forms of the vaccine to be equally protective.

The production of live attenuated vaccines is under investigation. The use of avirulent strains of *V. anguillarum* as live attenuated vaccines was tested by Norquist *et al.* (1989). Three avirulent mutants were able to induce protective immunity against the homologous as well as a heterologous strain of *V. anguillarum*. Also, these

attenuated strains were capable of inducing cross-reactive protective immunity against *A. salmonicida*. However, more needs to be known about the stability of these strains before they can be recommended for commercial use Norquist *et al.* (1989).

The feasibility of constructing live attenuated strains of *V. anguillarum* that might be useful in future vaccines has been anticipated (Singer *et al.*, 1991). However, Smith (1988) concluded that it is unlikely that *Vibrio* vaccines will become more sophisticated than the present simple formulations. Improvement in formulation of currently used vaccines by addition of water-soluble adjuvants to in order to improve potency and duration of protection of immersion-applied vaccines was seen as a need by Smith (1988).

For a *Vibrio* vaccine to be effective, it must contain the serotypes of the pathogen indigenous to the locality in which it is to be used. Since there is only one known *V. anguillarum* responsible for vibriosis in Tasmanian salmonids, a successful vaccine "Anguillvac-C" a formalin killed vaccine has been introduced to Tasmanian fish farmers. It is currently produced by the Fish Health Unit, Department of Primary Industry of Tasmania, under the supervision of Dr. Jeremy Carson. This vaccine has been successful and there is great demand for it (Carson, pers. comm.).

Cumulative percentage mortalities among experimental groups of fish vaccinated against vibriosis in experiments conducted by several researchers are given in Table 3.1.

Table 3.1: Cumulative percentage mortalities among experimental groups of fish vaccinated against vibriosis in experiments conducted by several researchers

Reported by	Fish species	Vaccine preparation	Days post-Immunisation (number of bacteria used in challenge)	% Mortality				
				Unvaccinated controls	Oral vaccinated	Immersion vaccinated	Injected vaccinated	Spray vaccinate
Baudin-Laurencin & Tangtrongpiros (1980)	Groups of fish	Formalin-killed	???(not stated)	33.8	31.7	2.1	1.4	not done(ND)
Amend & Johnson(1981)	Salmonids	"	30($1.6x10^7$)	52	27	4	0	1
Håstein & Refsti(1986)	Rainbow trout	Commercial formalin killed	75(ND)	68.7	ND	7	3	ND
Horne *et al.*, (1982)	"	"	56($2x10^7$)	100	36	ND	0	ND
	"	"	85 "	100	78	53	7	ND
	"	"	330 "	78	ND	56	ND	
Johnson & Amend(1983)	Chinook salmon	"	??	97	35% Oral intubation(69% with gelatin)	ND	ND	ND
Dec *et al*(1990)	Turbot	Commercial vaccine (Rhone-Merieux)		65.4	19.2	ND	ND	ND
	Sea bass			49.1	11.3			
Antipa(1976)	Chinook salmon	Formalized Heat-killed	1-150(Natural)	85.4	ND	ND	37.8	ND
			1-150(injection)		"	"	22.3	"

3.2 Experiment I Passive immunisation (vibriosis):

(determination of the rate of clearance of AVA from fish sera)

3.2.1 Introduction and Aims:

Passive immunisation of fish against vibriosis has been tried to prevent vibriosis (section 1.1 and Table 1.1.1). However, a detailed study to elucidate aspects of passive immunity at different intervals and monitoring the rate of clearance of Ig from fish sera by ELISA has not been conducted.

The aim of this study was to measure the rate of loss of transferred immune sera (anti-*Vibrio anguillarum* antibodies) from fish sera. Fish in two replicates were injected i.p. with one of three types of immune antisera (sheep, rabbit or fish).

3.2.2 Materials and Methods

Immune sera:

Anti-*Vibrio anguillarum* antisera of sheep, rabbit and fish were used in which the level of anti-*Vibrio* antibodies had been determined by ELISA (sections 2.4, 2.6.1).

Fish:

Rainbow trout: In this experiment 92 rainbow trout [(126± 9.5 g (mean ± S.D. of weight)] were randomly allocated to different groups. Fish were tagged and injected i.p. with 0.1 ml 100 g^{-1} of fish body weight of three types of immune sera (either sheep, rabbit or fish). Twenty fish were allocated as controls and were bled at each interval (Table 3.2.1). The control fish were all tagged with plastic T-bar anchor tags (placed in the flesh just below the base of the dorsal fin for individual identification) and maintained in 4000 L tanks at 15°C.

Table 3.2.1: Experimental design for monitoring the rate of clearance of passively transferred anti-*V. anguillarum* antibodies (AVA) from rainbow trout sera

Treatment	Route of administration of AVA	No. of fish	Number of fish sampled at each time							
			2h,	4h,	6h,	24h	3w	5w	8w	10w
Sheep AVA (ELISA O.D; 2.503)*	i.p.	24	6	6	6	6	12	12	12	12
Rabbit AVA (ELISA O.D; 1.486)*	i.p.	24	6	6	6	6	12	12	12	12
Fish AVA (ELISA O.D; 1.204)*	i.p.	24	6	6	6	6	12	12	12	12
Control (Untreated) (ELISA O.D; 0.134)*	-	20	-	-	-	-	10	10	10	10
			(Total No. fish in 2 replicates= 92)							

h= Hours post-immunisation (p.i.) m= month
w= Weeks " " *= Optical density at 405 nm

Six fish from each group were bled at each interval (2 h, 4 h, 6 h, 24 h) in such a way that by 24 h all immunised fish were bled. Twelve fish in each group were bled at week 3, 5, 8, or 10.

Atlantic salmon: In order to measure the rate of clearance of immunoglobulins of sheep, rabbit and fish in Atlantic salmon sera a similar experimental design was used, 80 fish (mean weight 96± 10.2g)were used. Fish were divided in four groups; control, sheep AVA injected, rabbit AVA injected and fish (rainbow trout) AVA injected. Control fish were only tagged but other groups received 0.1 ml 100 g^{-1} body weight of immunised sera intraperitoneally. Atlantic salmon were sampled only at week 1 and 4 post-immunisation.

ELISA:

Sheep, rabbit and fish ELISAs were conducted to measure the antibody level of the antisera transferred to fish as described in section 2.6. For the sheep and rabbit AVA ELISAs soluble antigen was used to coat ELISA plates. Whole cell antigen was used to coat the plate in the ELISA for measuring the level of fish AVA.

3.2.3 **Results:**

Rainbow trout:

Administration of sheep anti-*Vibrio* antibodies was followed by a significant elevation of these antibodies in fish sera 2 h post-immunisation. Sheep AVA antibody levels declined slightly at 4 and 6 h and before nearing a plateau between 6 h and 5 weeks post-immunisation (Figure 3.2.1). These antibodies declined to the mean ELISA O.D of 0.300 at week 8, and O.D of 0.252 at week 10 (Table 3.2.2). There was no significant difference between the sheep AVA antibody level at 6h, week 3 and week 5 ($P< 0.05$), however these antibodies reduced significantly in fish sera 5 weeks post-immunisation. The ratio of O.D of immunised fish with sheep AVA to O.D of non-immunised fish changed little up to week 5 (Table 3.2.3).

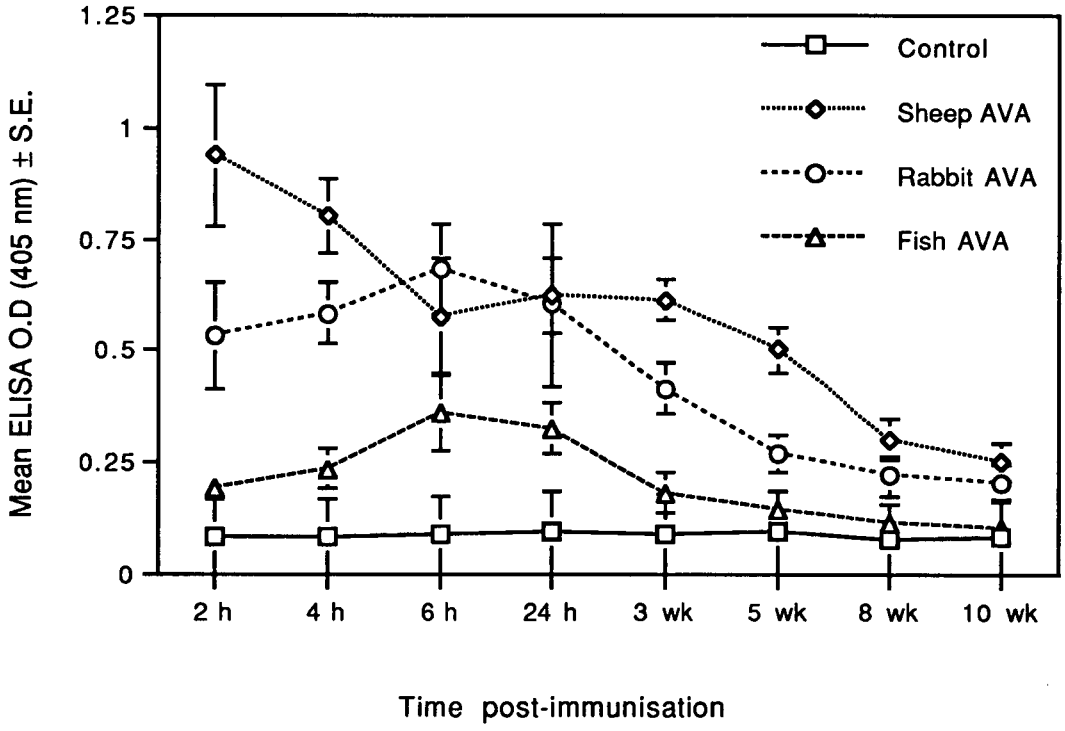


Figure 3.2.1: Rate of clearance of 3 types of anti-*V. anguillarum* antibodies from rainbow trout sera, 2 hours up to 10 weeks post-immunisation

Fish given rabbit anti-*Vibrio* antibodies showed a significant elevation in serum antibody level 2 h post- immunisation. Antibody level continued to increase 6 h after immunisation and then declined. At week 10 the mean ELISA O.D was 0.201 which was still significantly higher than that of control fish (Table 3.2.2). The ratio of O.D of fish immunised with rabbit AVA to non-immunised fish was 4.651 up to 3 weeks post-immunisation (Table 3.2.3).

Fish passively immunised with fish anti-*Vibrio* antibodies had increasing levels of AVA antibodies 6 h post-immunisation (O.D= 0.359). These antibodies then declined significantly and mean ELISA O.D at week 10 was the same as O.D of

control fish (Figure 3.2.1). The highest ratio of O. D of immunised fish to those of control fish was at 6 h post-immunisation. This ratio then declined (Table 3.2.3).

Table 3.2.2: The summary of figure 3.2.1; rate of clearance of AVA from fish serum (shown by O.D= antibody level)

Time post-passive Immunisation	6h	Week 3	week 5	week 8	week 10
	ELISA O.D readings (mean ± 1 S.E.)*				
Control	$0.087^{a}\pm0.048$	$0.089^{a}\pm0.067$	$0.093^{a}\pm0.026$	$0.078^{a}\pm0.04$	$0.083^{a}\pm0.040$
Sheep AVA	$0.574^{bc}\pm0.132$	$0.612^{b}\pm0.043$	$0.501^{bd}\pm0.051$	$0.300^{c}\pm0.044$	$0.252^{c}\pm0.042$
Rabbit AVA	$0.682^{b}\pm0.100$	$0.414^{d}\pm0.058$	$0.267^{c}\pm0.044$	$0.219^{c}\pm0.045$	$0.201^{c}\pm0.024$
Fish AVA	$0.359^{c}\pm0.087$	$0.180^{a}\pm0.047$	$0.144^{a}\pm0.022$	$0.112^{a}\pm0.042$	$0.099^{a}\pm0.036$

*Values with the same superscript were not significantly different ($P< 0.05$).

Table 3.2.3: The ratio of O. D of immunised fish relative to O. D of non-immunised fish (control) at various intervals post-immunisation

Time post-passive Immunisation	6h	Week 3	week 5	week 8	week 10
Sheep AVA	6.597	6.786	5.387	3.846	3.036
Rabbit AVA	7.839	4.651	2.870	2.807	2.421
Fish AVA	4.126	2.022	1.548	1.435	1.192

Atlantic salmon:

The results of the passive immunisation of Atlantic salmon are shown in Figure 3.2.2. Sheep AVA was detected at high levels after one week post-immunisation and after 4 weeks it showed an O.D of 0.621 suggested the remarkable level of this antiserum in Atlantic salmon sera.

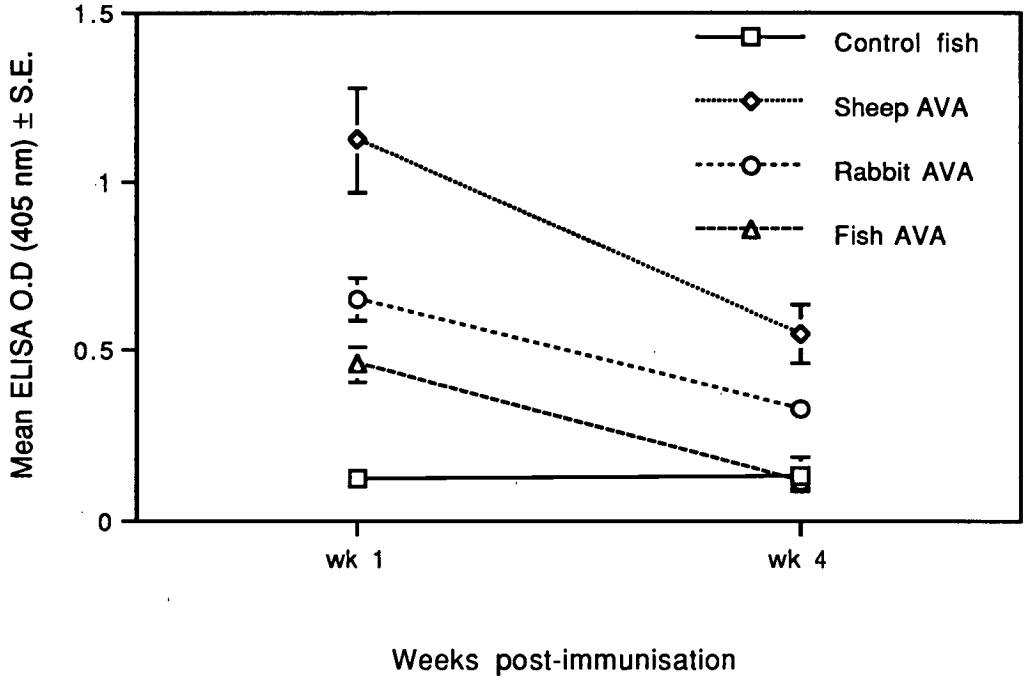


Figure 3.2.2: Rate of clearance of 3 types of anti-*Vibrio* antibodies from Atlantic salmon sera

Rabbit AVA in Atlantic salmon sera appeared with an O. D of 0.712 after 1 week and still significant level at week 4. Atlantic salmon sera showed significant rainbow trout AVA only at one week post-immunisation.

3.2.4 Discussion:

In this experiment, sheep anti-*Vibrio* antibodies reached peak levels in fish serum by 2 h whereas rabbit and fish AVA peaked at 6 h. A dip in the level of sheep AVA at 6 h post-immunisation could be due to the fact that different fish were bled at each time (up to 24 h post-immunisation).

The persistence of sheep AVA after administration was higher as compared with rabbit AVA. This is presumably due to the higher antibody level of the sheep AVA inoculum (Table 3.2.1). Sheep and rabbit antibodies transferred into fish consist mainly of IgG and have different catabolic rate from fish antibodies. IgG, which under normal conditions is found in the highest concentration of the five classes in normal individuals (human), generally shows the longest half-life (Table 3.2.4) (Waldmann *et al.*, 1976). Therefore, the longevity of sheep AVA in fish sera could be due to such

long half life of IgG. Lower metabolism of Ig in a poikilothermic condition of fish could be another reason for such a longevity.

Table 3.2.4: Metabolic properties of human immunoglobulins (normal individual), adapted from Waldmann *et al.* (1976)

Immuno-globulin	serum concentration (mg ml^{-1})	Distribution (% Intra-vascular of total body pool	Total circulating pool (mg kg^{-1})	FCR*	Half-life (days)	Synthtic rate mg kg^{-1}/day
IgE	0.0001	41	0.004	94.3	2.7	0.0038
IgD	0.023	75	1.1	37.0	2.8	0.4
IgM	0.93	76	37.0	18.0	5.1	6.7
IgA	2.6	42	95.0	25.0	5.8	24.0
IgG	12.1	45	494.0	6.7	23.0	33.0

*FCR= % of the intravascular pool catabolised per day.

Fish injected with fish AVA had an antibody peak at 6 h which declined afterwards. This is consistent with Groberg *et al.* (1979) who found that absorption of radiolabelled coho salmon Ig protein from the peritoneal cavity is relatively fast and is complete in 8 h at 12°C and the efficiency and kinetics of intraperitoneal absorption of Ig is temperature dependent. Immunised fish with fish AVA showed significantly lower amounts of antibodies post-immunisation than fish given sheep or rabbit AVA. This could also be due to the lower level of fish antibodies transferred to fish. Moreover, catabolic rate studies showed that normal tetrameric Ig of coho salmon has a relatively short half-life of ~ 49 h at 12°C (Voss *et al.*, 1980).

Another explanation for the differential results with sheep, rabbit and fish AVA include more rapid metabolism of rabbit and fish Ig compare with sheep Ig, or a more vigorous immune response against the former antigens (AVA as antigen for fish).

Atlantic salmon immunised by the same amount of the three types of antisera showed slightly higher level of antibody level in their sera 1 week and 4 weeks post-immunisation in comparison with rainbow trout. It is possible that differences in weight of rainbow trout (126 g) and Atlantic salmon (96 g) could account for higher ELISA O.D. However, these differences were not significant ($P< 0.05$).

Study of catabolic rate of mammalian antibodies and fish antibodies in fish sera using radioiodination of Ig, might clarify the observations made in this experiment.

3.3 Experiment II Adaptive humoral response of fish to sheep and rabbit AVA

3.3.1 Introduction and Aims:

In passive immunisation, heterologous proteins are transferred to fish. It was assumed that the fish immune system would recognise these antigens and produce antibodies. This experiment was set up to study the fish humoral response to sheep and rabbit sera containing IgG, serum proteins and other components. Therefore, sera collected at different intervals from fish immunised with sheep and rabbit AVA were tested by ELISA to monitor the level of antibody production against the mammalian sera.

3.3.2 Materials and Methods:

Sera:

The sera collected from rainbow trout in experiment I (section 3.2) were used. These sera were collected from 2 h up to 10 weeks after passive immunisation. Since the collected sera were mainly used for ELISA (in duplicate wells and repeating the test because of high coefficient of variation), some of these sera were pooled to allow the required volume in the ELISA in this experiment. Thus, the number of sera examined in this experiment (shown in Table 3.3.1 and 3.3.2) decreased over the time.

ELISA:

An ELISA was set up to determine the fish Ig against sheep and rabbit AVA components as described in section 2.6.3.2.

Fish given multiple doses of mammalian AVA:

Forty rainbow trout (mean weight 105 g) in two groups were injected with three doses of 0.1 ml either sheep (20 fish) or rabbit (20 fish) at intervals of two weeks. This was to observe if any death due to hypersensitivity could be demonstrated.

3.3.3 **Results:**

Fish response to sheep AVA:

The number of fish that responded to sheep AVA at different times are shown in table 3.3.1. A relatively small number of fish responded to sheep AVA. None of the control fish showed positive O.D measured by ELISA.

Table 3.3.1: Number and percentage of rainbow trout responded to heterologous sheep anti-*V. anguillarum* serum

Time post-passive Immunisation	Week 3	Week 5	Week 8	Week 10
	Number of fish and per cent			
Number of fish	n(%)	n(%)	n(%)	n(%)
+ fish anti-sheep	0 (0)	3 (12.5)	2 (12.5)	2 (16.6)
- fish anti-sheep	32	21	14	10
Total number of fish	32	24	16	12

Fish antibodies to sheep serum (with positive ELISA O.D) peaked at week 8 and declined after 8 weeks of immunisation (Figure 3.3.1). Control tests confirmed the binding attachment of sheep AVA and rabbit AVA to microtitre plates.

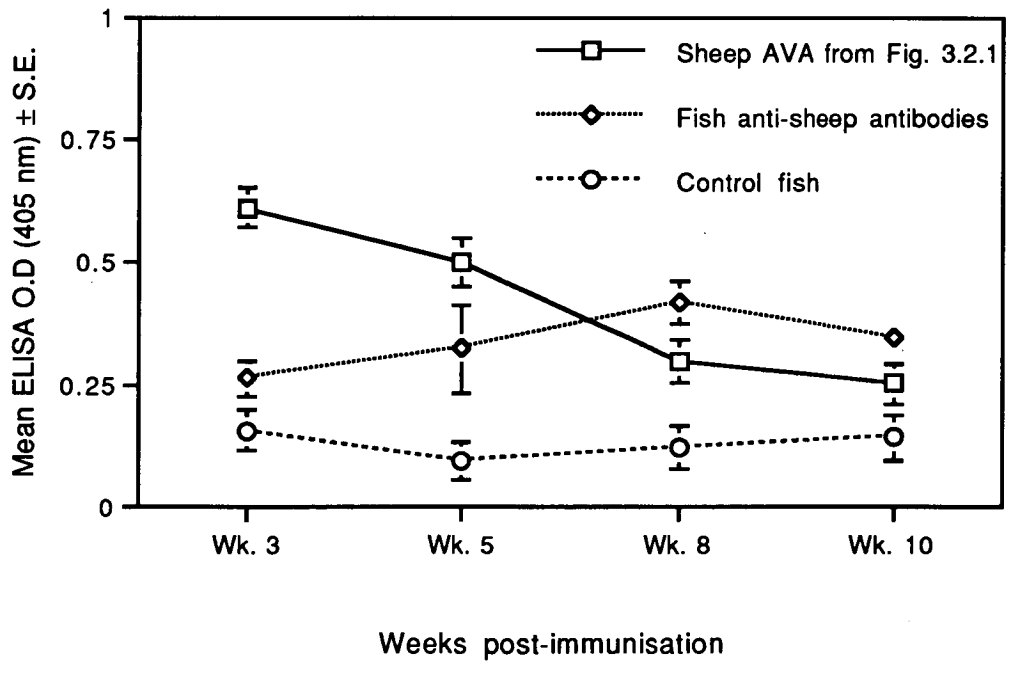


Figure 3.3.1: Rainbow trout humoral response to the heterologous sheep AVA

The number of fish which responded to the heterologous rabbit Ig are shown in table 3.3.2. A higher percentage of fish showed anti-rabbit antibodies when compared with fish injected by sheep AVA (Table 3.3.2).

Table 3.3.2: Number and percentage of rainbow trout responded against heterologous rabbit anti-*V. anguillarum* serum

Time post-passive Immunisation	Week 3	week 5	week 8	week 10
	Number of fish and per cent			
Number of fish	n(%)	n(%)	n(%)	n(%)
+ fish anti-rabbit	9(27.3)	9(34.7)	8(33.3)	4(30.8)
- fish anti-rabbit	24	17	16	9
Total number of fish	33	26	24	13

The antibody response of fish to the rabbit Ig is plotted in figure 3.3.2. The rabbit AVA decreased as the fish anti-rabbit Ig increased. Fish anti-rabbit Igs peaked at week 8 (ELISA O.D= 0.576) and gradually declined. Fish responded to the rabbit antiserum from week 3 and the reaction continued to rise up to week 8.

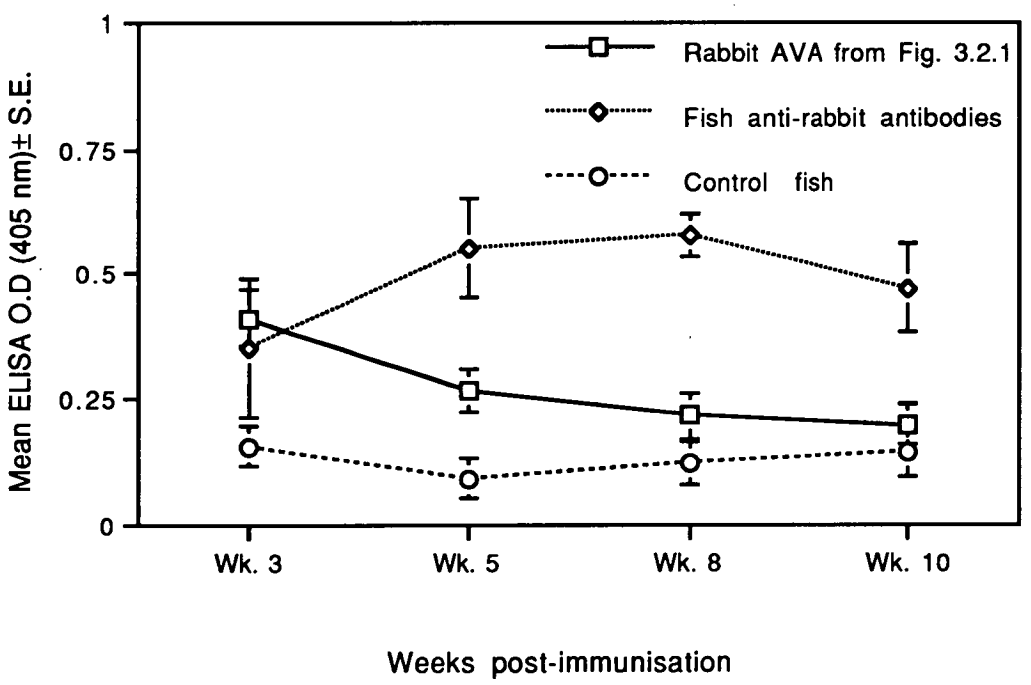


Figure 3.3.2: Rainbow trout humoral response to the heterologous rabbit AVA

Table 3.3.3: Summary of figures 3.3.1 and 3.3.2 showing ELISA O.Ds ±S.E.

Weeks post-immunisation	Control fish	Fish injected i.p. with Sheep AVA	Fish injected i.p. with Rabbit AVA
Wk. 3	0.158±0.041	0.264^{a}±0.035	0.352^{a}±0.140
Wk. 5	0.094±.038	0.323^{a}±0.089	0.551^{b}±0.098
Wk. 8	0.124±.044	0.418^{c}±0.042	0.576^{b}±0.042
Wk. 10	0.143±0.045	0.345^{a}±0.021	0.473^{bc}±0.087

Values with the same superscript are not significantly different ($P<0.05$).

Fish given multiple doses of mammalian AVA

Non of the forty fish injected intraperitoneally with either sheep or rabbit AVA died or exhibited apparent anaphylaxis after 3 injections in two weeks intervals.

3.3.4 **Discussion:**

In this experiment, fish adaptive immune responses to rabbit AVA were significantly higher than those against sheep AVA ($P< 0.05$) (Table 3.3.3). It is possible that at the molecular level rabbit proteins were more antigenic than sheep AVA. Although, rabbit AVA declined more steeply than sheep AVA possibly due to rapid metabolism in fish liver or spleen (section 3.2.3), fish adaptive immune responses was higher to these antibodies. It is probable that such a response from fish to rabbit AVA induces the clearance of these antibodies as it was seen from the results of rate of decay (Figure 3.2.1).

Fish might respond to other serum proteins as well as the antibodies. These hypothesis could be clarified if affinity purified Igs was used. In that case one could see if the fish responses were against heterologous Igs or other sera components. Further studies are required to clarify if this response is a significant modulator of the persistence of mammalian immunoglobulins in fish.

None of the fish injected with multiple doses of hyperimmune sera of sheep and rabbit AVA died as a result of hypersensitivity. It can be concluded that these antibodies are safe at therapeutic and prophylactic doses to fish, even in multiple injections.

3.4 Experiment III: Active immunisation (vibriosis)

3.4.1 Introduction and Aims:

As it was discussed in section 3.1.8, bacterins work ideally in control of vibriosis worldwide. This experiment aimed to compare production of active antibodies with the persistence of passive antibodies. The humoral responses of fish to a complex particulate bacterial antigen, and an adjuvanted vaccine were investigated. This experiment evaluated the antibody responses of rainbow trout actively immunised concurrently with fish in passive immunisation experiment (section 3.2) to compare the persistence of fish antibodies itself and sheep and rabbit Ig transferred to fish.

In order to compare the antibody responses of Atlantic salmon, 20 Atlantic salmon were also immunised with the same vaccines as rainbow trout.

3.4.2 Materials and Methods:

Bacterial antigen:

Bacterins for active immunisation of fish were prepared as described in section 2.2. The inoculum for intraperitoneal injection of fish in this experiment contained 1 mg (dry weight) bacterin.

Fish:

Rainbow trout: Experimental fish (126± 11.0 g) were allocated randomly in two replicates in two tanks. Fish were anaesthetised, injected intraperitoneally either with 0.1 ml of *V. anguillarum* , *V. anguillarum* in FCA or immersed into *V. anguillarum* immersion solution at week 0 (Table 3.4.1). Fish immunised by immersion were placed in a 30 L fish bin containing a 1:10 dilution of vaccine containing $1x10^{10}$ cells of formalin-killed *V. anguillarum* whilst netted for 30 seconds. They were then held in another fish bin with freshwater for 30 seconds then anaesthetised, tagged (placed through the base of the dorsal fin in the flesh until it penetrates between the interneural spines) and returned to the main tank. All fish were individually tagged simultaneously (wk 0) at the time of immunisation. Fish in the control group did not receive any injection or immersion vaccination.

Fish were kept in 4000 L circulating, temperature controlled tanks at 15°C.

Table 3.4.1: Experimental design for active immunisation of rainbow trout

Groups	Number of fish	Immunogen given at week 0	Route of administration
Control	28		
V. anguillarum	14	Formalin killed cells	i.p.
V/FCA	14	Formalin killed cells in FCA	i.p.
V. anguillarum	14	Formalin killed cells	Immersion

At week 3, 5, and 8 fish were bled and sera were collected as described previously.

Atlantic salmon: Twenty Atlantic salmon (110± 7.4 g) were used to study the humoral response of these fish. Fish were immunised at week 0 and bled 4 and 6 weeks post-immunisation.

ELISA:

The ELISA protocol used to determine fish antibody responses was as described in section 2.6.3.1.

3.4.3 **Results:**

Rainbow trout: Significant elevation in antibody level was present at week 3 in all groups that had been immunised with *V. anguillarum* vaccines at week 0, but the greatest response was present where the antigen had been administered with the adjuvant. Antibody levels continued to increase in following weeks in all group except of the immersion immunised group. Thus, the persistence of antibody appeared to be related to the method of immunisation, being least with immersion immunisation and greatest with i.p. immunisation with the adjuvanted vaccine.

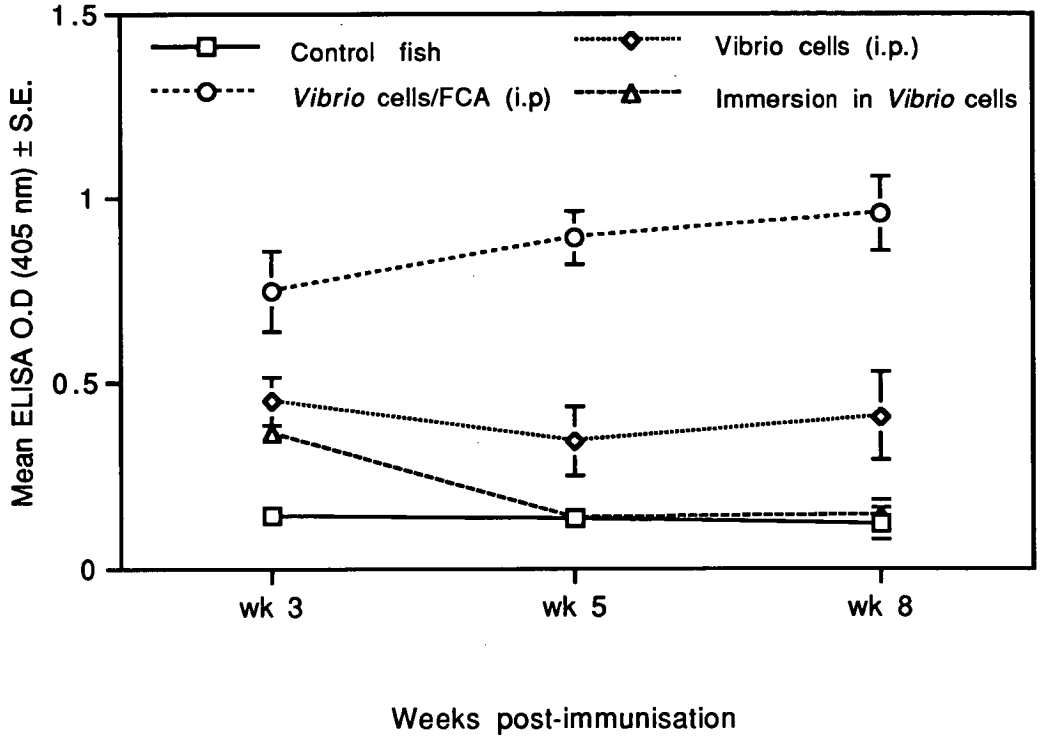


Figure 3.4.1: Rainbow trout humoral response to different antigens with or without adjuvant at different times post-immunisation

Serological responses (a summary of ELISA O.D of Figure 3.4.1) of rainbow trout to different immunogens are shown in Table 3.4.2.

Table 3.4.2: Summary of Table 3.4.1, humoral responses of fish to different immunogens

	(ELISA O.D readings± S.E. mean)			
Time (p.i.)	Week 0	Week 3	week 5	week 8
Control	0.145^{a}±0.017	0.152^{a}±0.016	0.160^{a}±0.019	0.143^{a}±0.014
V. angu (i.p.)	_	0.452^{b}±0.066	0.342^{b}±0.096	0.409^{b}±0.119
V. angu./FCA (i.p.)	_	0.747^{c}±0.111	0.890^{d}±0.71	0.959^{d}±0.101
V. angu (Immersion)	_	0.363^{b}±0.028	0.108^{a}±0.015	0.127^{a}±0.020

Values with the same superscript are not significantly different ($P< 0.05$).

In comparing the result of passive immunisation (section 3.2.3) and this result, it is obvious that at week 5 the humoral response of fish to *V.anguillarum* /FCA increased while the amount of sheep AVA reduced (Figure 3.4.2). There were no statistical differences ($P< 0.05$) between antibody level of fish injected with *Vibrio* bacterin/FCA and fish injected with sheep AVA at week 3 post-immunisation. Also, there was no significant difference between 1) sheep AVA level and fish AVA (due to injection of *Vibrio* cells) in fish sera up to 8 weeks post-immunisation, 2) rabbit AVA level in fish sera with those antibodies that fish actively produced after 5 weeks post-vaccination with *Vibrio* cells.

The persistence of passive antibodies and production of active antibody levels (This is a compilation of data already presented to aid interpretation) is shown in figure 3.4.2.

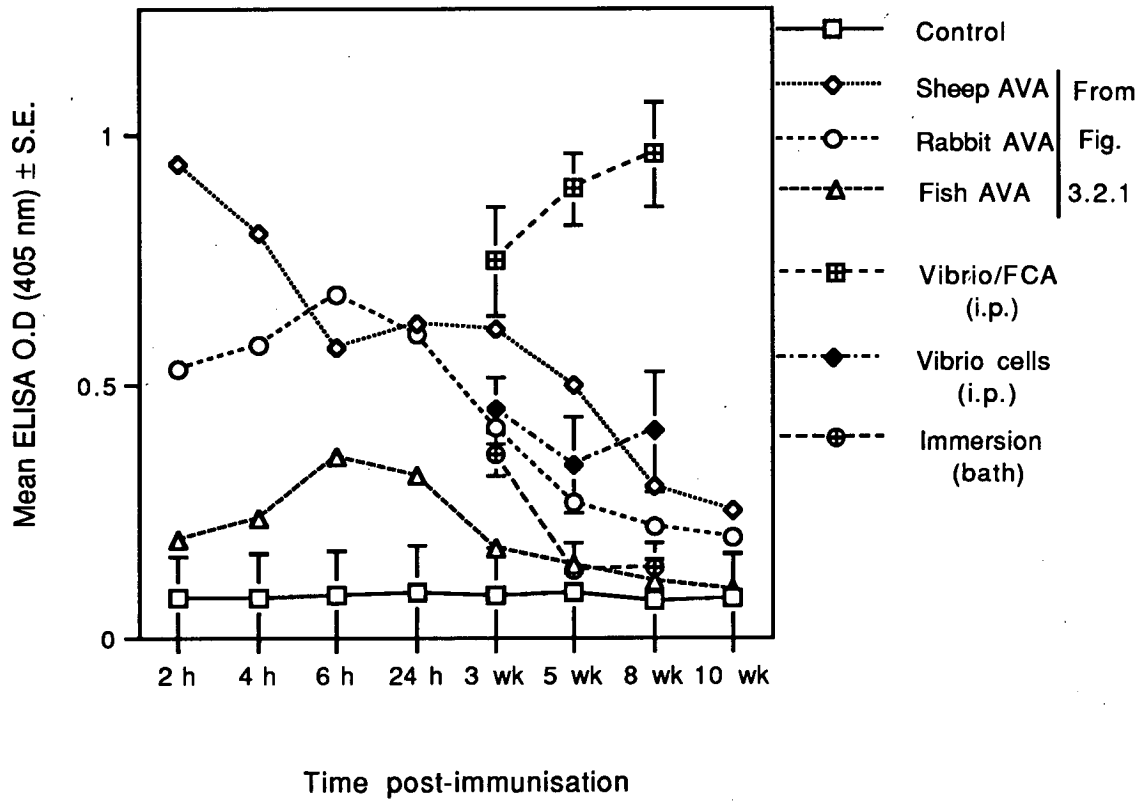


Figure 3.4.2: Comparison of the persistence of AVA in passively and actively immunised rainbow trout

Thus, antibodies due to i.p. immunisation of fish were elevated from 3 weeks while they declined from 3 weeks in those fish immunised by immersion. Generally passively transferred antibodies declined from 5 weeks post-immunisation onward.

Atlantic salmon: Results of the humoral responses of twenty Atlantic salmon immunised with *V. anguillarum* cells/FCA showed a significant elevation of fish antibodies from one week up to 6 weeks post-immunisation (Figure 3.4.3).

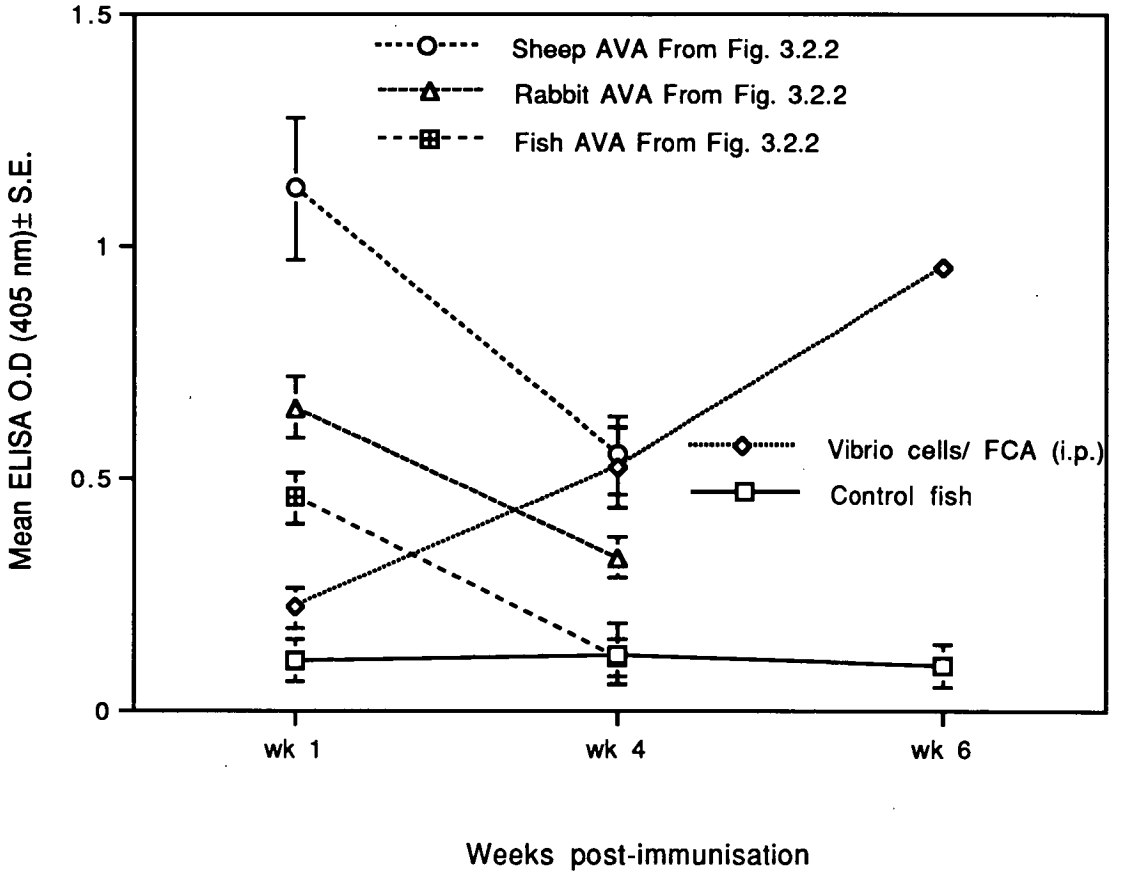


Figure 3.4.3: Atlantic salmon humoral response to *V. anguillarum* cells compared with the rate of clearance of sheep, rabbit or rainbow trout AVA from Atlantic salmon sera

3.4.4 Discussion:

When comparing active and passive immunisation, it is apparent that passive immunisation gives an immediate antibody level. This appears 2h post-immunisation and may last for 10 weeks or more (Figure 3.2.1). Active immunisation, in contrast, does not seem to appear in less than 3 weeks. For example, Atlantic salmon sampled after one week of immunisation showed an antibody level (mean O.D of 0.223) (Table 3.4.3) which was significantly different when compared to controls ($P< 0.05$). Also the pattern of elevation of antibodies in fish following immunisation with FCA containing and non-adjuvanted vaccines showed less antibody level at early weeks after immunisation. Therefore, it can be concluded that one week is the minimum required for development of immunity even though the level of antibodies produced may not sufficient to combat the pathogen.

Fish immersed in *Vibrio* cells, had elevated antibody level (O.D= 0.363) at three weeks post-immunisation. At week 5 and later no anti-*V. anguillarum* antibodies could be detected. However, this is in agreement with Whittington *et al.* (1994) who reported a significant elevation in antibody level 3 weeks after bath vaccination with

V. anguillarum but levels decreased thereafter. Immersion vaccination provides protective immunity for up to one year (Alexander, 1990). Despite low or no detectable antibody production after one month, the immunity seems presumably to be the resultant of enhanced cell mediated defense mechanism in fish, or immune memory with rapid humoral responses after contact with the pathogen.

When comparing ELISAs O.D, it is worth considering the endogenous antibody levels (fish response) after active immunisation and rabbit/ sheep AVA transferred to fish and the problem in making a conclusion because the ELISA may not have equal signal for each mole of antibody. However, protective immunity conferred with i.p. of sheep, rabbit or fish AVA (section 3.6) answers the problem to some extent.

Atlantic salmon responded in a similar manner to rainbow trout. The monoclonal antibody used for detection AVA in Atlantic salmon was anti-rainbow trout IgM, however the Atlantic salmon responses were detected indicating a useful cross reaction that can be expected in future studies.

3.5 Experiment VI Comparison of the efficacy of delivery of antiserum at two different sites of intraperitoneal injection

3.5.1 Introduction and Aims:

Intraperitoneal injection has been used in fish for decades to deliver chemotherapeutics for disease treatment and vaccines for immunisation programs. It has been used in the Pacific north-west of the USA to vaccinate Pacific salmon against vibriosis since the early 1970s and, more recently, the method has become common practice in Scandinavia for furunculosis prophylaxis, since injection vaccination proved to be the most effective(Smith, 1988; Erdal and Reitan, 1992). Intraperitoneal injection is relatively safe and simple even in very small fish. It has been described as the most effective way of immunising fish, though the method usually requires anaesthetisation before handling. The use of adjuvants which enhance the magnitude of the immune response is also possible with this method (Ellis, 1988a).

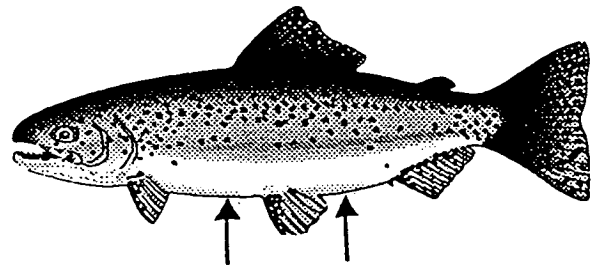
Despite the importance of this route of delivery of drugs and vaccines there is little published information on the exact site of injection to be used. It has been suggested for ornamental fish that midway between the pelvic fin and anus is the appropriate site for intraperitoneal medication (Stoskopf, 1988). Similarly, Ashburner (1978) and Reddacliff (1985) described a site just anterior to the vent. Others (Whittington and Cullis, 1988; Nilsen *et al*, 1992; Turgeon and Elazhary, 1992) have used a site immediately anterior to the pelvic fins. However, none of these workers appear to have critically evaluated the appropriateness of the various sites of injection.

During studies involving rainbow trout, when fish were injected intraperitoneally using the site midway between pelvic fin and anus , it was observed that some inocula had been injected into the rectum. Consequently, it was decided to investigate the efficacy of injecting material at two different sites.

3.5.2 Materials and Methods:

Fish:

In the experiment reported below, Seventy two rainbow trout (880± 22.2 g) in two groups were passively immunised with 0.4 ml sheep anti-*V. anguillarum* antiserum. Thirty six fish in group 1 were injected at site 1 (midway between the pelvic fin and anus on the midline), and 36 fish in group 2 were injected with the same amount of antiserum at site 2 (1- 1.5 cm anterior to pelvic fin, on the midline) (Plate 3.5). Fish were injected with 19 mm length and 25 gauge needles.



Site 2 Site 1

Plate 3.5: Two different sites of intraperitoneal injection

Fish were individually tagged and maintained in a temperature-controlled tank(17°C) and fed with commercial trout pellets. All injections were made by one, experienced person.

ELISA:

Fish were bled one week and four weeks after injection by caudal vein. The rates of loss of sheep anti-*Vibrio* antibodies from the serum in two groups of fish were monitored by sheep ELISA (section 2.6.1). The test was undertaken in duplicate wells for each sample and also it was conducted at two different laboratories (Mt. Pleasant Laboratories and EMAI immunology Laboratory, where the ELISA's were performed).

3.5.3 **Results:**

Figure 3.5 shows the results of the ELISAs. There were no significant differences ($P < 0.05$) in the amount of AVA antibodies comparing the two sites of injection over time. Also no significant differences were observed comparing the results between the laboratories ($P < 0.05$).

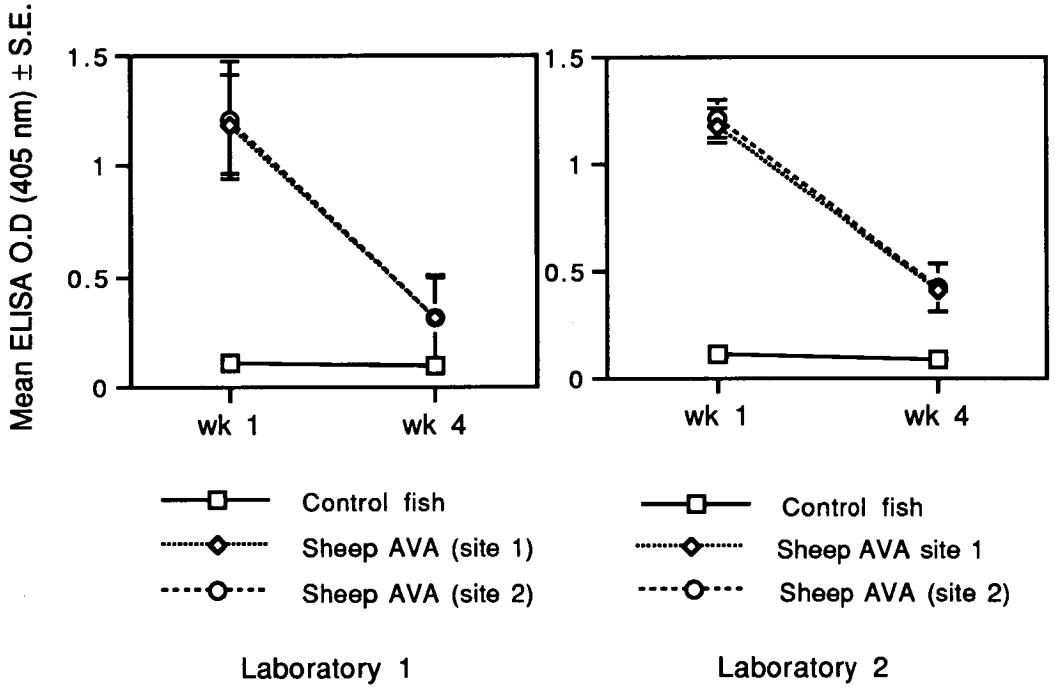


Figure 3.5: Levels of sheep AVA in rainbow trout sera measured by ELISA in two different laboratories

3.5.4 **Discussion:**

This experiment shows that i.p. injection of fish at both sites 1 and site 2 gave similar efficacy. Also regarding similar results obtained by ELISA between the two laboratories, both used the same method for ELISA assay and as pointed out earlier in section 2.6 for optimisation of ELISA, transferring the method from one laboratory to another was successful. It can be concluded that once an experienced practitioner understands the basic anatomy of fish either of these sites is appropriate for i.p. injection. Though site 1 has the disadvantage of potentially injecting into the rectum, it minimises the chance of injuring the liver, spleen or kidney. Site 2, however could cause damage to the pyloric caeca, liver or spleen.

The following protocol is recommended: Position the fish on it's back inside a sponge slit lined with plastic, to minimise stress. Use a gauge of needle as small as possible and appropriate to the solution to be injected. With a controlled insertion penetrate through the skin and muscle to a depth slightly greater than the thickness of wall of the abdomen. Rub the injection site after every injection to disperse the injected solution and minimise the chance of losing the drug or vaccine after the needle is removed.

3.6 Experiment V Evaluation of protection (vibriosis)

3.6.1 Introduction and aims:

Levels of protection in passively immunised fish (Table 1.1.1, section 1.1) and actively immunised fish determined by other researchers were discussed previously (Table 3.1, section 3.1.8). This experiment was carried out in order to evaluate the protective efficacy of anti-*Vibrio anguillarum* antibodies one, two and three months post-immunisation. An experiment (different from experiment I) was conducted specifically to evaluate protection in the knowledge that passive antibody levels could be high at 1 month and declined during the time (experiment I). In this experiment, fish were immunised passively with three different antisera at the time 0 and challenged with virulent *V. anguillarum* (LD_{70}). One extra groups of fish were immunised actively to compare results of passive and active immunisation.

Little work has been done regarding protective value of passive immunity against vibriosis. This new approach can provide passive transfer of antibodies and can possibly be applied against other infectious diseases of fish. The main aim of this experiment was to compare passive and active protective immunity concurrently at a high challenge level (LD_{70}) and evaluate the protective immunity conferred to fish by passive immunisation.

3.6.2 Materials and methods:

Fish:

Rainbow trout (average weight of 70± 5.3 g) were acclimatised to temperature controlled 4000 L tanks at the trout room before immunisation. Fish were starved for 12h prior to immunisation. Fish were allocated randomly to 5 groups in two replicates (Table 3.6.1).

Antisera for passive immunisation:

Anti-*Vibrio anguillarum* antibodies (AVA) for passive immunisation of fish were prepared for use in this experiment as described in section 2.4. Each fish while lightly anaesthetised received 0.1 ml 100 g^{-1} fish body weight i.p. of sheep, rabbit, or fish AVA. The antibody levels (ELISA O.Ds) of sheep and rabbit (at dilution of 1: 200) or fish AVA (at dilution of 1: 100) used for passive immunisation of fish were 2.503, 1.486, 1.2 respectively.

Bacterin for active immunisation:

Formalin killed *V. anguillarum* for immersion vaccination of fish was prepared as described in section 2.2.2. Undiluted vaccine contained 1x 10^{10} cells. Fish were held in a 1: 10 dilution of vaccine (contained 5.5 x 10^9 ml^{-1}) whilst netted for 30 seconds in a aerated 20 L fish box (18 L freshwater + 2 L undiluted vaccine).

Virulent *V. anguillarum* with LD_{70} for challenge:

Fish were challenged with the virulent bacteria as described in section 2.10.1. In this experimental challenge, fish were inoculated with $6.8x10^6$ cfu 0.1 ml^{-1} (LD_{75}), $8x10^6$ cfu 0.1 ml^{-1} (LD_{80}) $6.2x10^6$ cfu 0.1 ml^{-1} (LD_{73}) of live *V. anguillarum* cells at one month, two months and three months challenges respectively.

Table 3.6.1: Experimental design for challenge of passively and actively immunised fish in an experimental infection of *V. anguillarum*

Treatment*	Route of administration	Number of fish challenged(in two replicates)		
		1m	2m	3m
Sheep AVA	i.p.	20	20	30
Rabbit AVA	i.p.	20	20	30
Fish AVA	i.p.	20	20	30
Immersion (bath)	-	20	20	30
Control (tagged, untreated)	-	40	20	20
			Total No. of fish= 360	

* each fish was injected with 0.1 ml $100g^{-1}$ body weight

AVA= Anti-*V anguillarum* antisera m= months post-immunisation

Challenge:

Immunised fish (both passively and actively) in two replicates were challenged with *V. anguillarum* (experimental design, Table 3.6.1). Control fish (in two replicates) that had not received any treatment and were only tagged at the time of immunisation, were included in the challenge. Twenty of the control fish (out of 40 fish) were injected with sterile saline at the time of one-month challenge in order to ascertain if any deaths were due to i.p. injection procedure. These fish were kept in the trout room.

Before challenge, the required number of fish for each challenge were taken to an isolated (disease) unit and kept in freshwater tanks at 15°C, acclimatised for 2 days and then challenged.

Fish were challenged with 0.1 ml i.p. *V.anguillarum*. The temperature was maintained at 15°C. Fish were observed twice daily for 14 days after challenge.

Freshly dead and moribund fish from each group were collected and their kidneys inoculated aseptically on to blood agar for the isolation of *V. anguilarum*. The isolated bacteria were recultured if it was required and confirmed both morphologically and serologically with rabbit AVA as *V. anguillarum*.

Mortalities began after approximately 48 h and ceased 5 days later. Activity, response to feeding, development of lesions and mortalities were recorded.

3.6.3 **Results:**

One-month challenge:

Results of challenge of immunised fish allocated for one month were reported as number of mortalities and relative per cent of survival (RPS, mean of two replicates' RPSs± S.E.) (Table 3.6.2).

Table 3.6.2: Mortality and RPS after challenge(with virulent *V. anguillarum*, LD_{75}) of rainbow trout immunised passively (sheep, rabbit, and fish AVA) and actively (immersion) at one month post-immunisation (p.i) as compared to controls

Treatment	Number of mortality caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicate	RPS ± S.E.
Sheep AVA	1/10	0/10	1/20	93.3 ± 6.67
Rabbit AVA	1/10	1/10	2/20	86.6 ± 0.0
Fish AVA	4/10	5/10	9/20	40.0 ± 6.66
Active Immunisation (immersion)	1/10	1/10	2/20	86.6 ± 0.0
Controls				
Challenged non-immune	8/10	7/10	15/20	0.0 ± 6.66
Non-challenged non-immune	0/10	0/10	0/20	100 ± 0.0

* Experimental challenge $LD_{75}=1.2x10^7$ cfu 0.1 ml^{-1} by i.p. injection.

Control groups, which were non-immunised fish, suffered a 75% mortality. In contrast, fish immunised with immune sera showed consistent and significant protection (chi-square; P= 0.005). Those immunised with fish serum, however, were only weakly protected (RPS of 40.0%) compared with fish immunised with sheep and rabbit AVA serum (RPS of 93.3 and 86.6% respectively).

Both passive and active (immersion) immunisation provided protection in comparison with control group but the highest RPS (93.3%) was observed in those fish immunised by sheep AVA.

The result of bacteriological examination of mortalities due to challenge showed 100% positive isolation of *V. anguillarum*. No *V. anguillarum* was isolated from the kidney of survivor fish 14 days post-challenge. None of the controls injected with PBS died post-injection.

Two-month challenge:

Results of the second challenge after two months of immunised fish allocated for this challenge are shown in table 3.6.3 .

Table 3.6.3: : Mortality and RPS after challenge(with virulent *V. anguillarum*, LD_{80}) of rainbow trout immunised passively (sheep, rabbit, and fish AVA) and actively (immersion) at two months p.i. as compared to controls

Treatment	Number of mortalities caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicate	RPS± S.E.
Sheep AVA	5/10	7/10	12/20	25.0 ± 12.0
Rabbit AVA	5/10	8/10	13/20	18.7 ± 18.75
Fish AVA	8/10	6/10	14/20	12.5 ± 12.50
Active Immunisation (immersion)	1/10	0/10	1/20	93.0 ± 6.25
Controls Challenged non-immune	8/10	8/10	16/20	0.0 ± 0.0

* Experimental challenge LD_{80}= $8x10^6$ cfu 0.1 ml^{-1} by i.p. injection

Protection in this trial ranged from RPS of minimum 25% (sheep AVA) to 18.7% (rabbit AVA) and 12.5% (fish AVA). RPS of immersion vaccination fish was 93% the highest of all for this challenge.

Comparing the results of first challenge after one month and the second challenge after 2 months post-immunisation, it is obvious that protection rate of immune sera declined markedly. However, immersion vaccinated fish provided fish a high protection (RPS= 93.0%) after two months post-immunisation.

No bacterial fish pathogens were detected in surviving fish (samples from kidney and brain).

Three-month challenge:

Results of the three months challenge are shown in table 3.6.4. In this challenge there were three replicates.

Table 3.6.4: : Mortality and RPS after challenge(with virulent *V. anguillarum*, LD_{73}) of rainbow trout immunised passively (sheep, rabbit, and fish AVA) and actively (immersion) at three months p.i. as compared to controls

Treatment	Number of mortalities caused by *V. anguillarum**				
	Rep. 1	Rep. 2	Rep. 3	Combined Replicates	RPS± S.E.
Sheep AVA	7/10	5/10	7/10	19/30	13.3 ± 9.13
Rabbit AVA	8/10	7/10	6/10	21/30	4.1 ± 7.90
Fish AVA	7/10	6/10	7/10	20/30	9.0 ± 4.56
active Immunisation (immersion)	2/10	1/10	1/10	4/30	81.8 ± 4.54
Controls Challenged non-immune	9/10	5/10	8/10	22/30	0.0 ± 16.45

* Experimental challenge LD_{73}= $6.2x10^6$ cfu 0.1^{-1} ml by i.p. injection.

The results indicated that immersion vaccination provided almost complete protection while protection obtained by intraperitoneal administration of immune sera after three months post-immunisation was only marginally effective.

Sheep AVA apparently marginally protected fish 3 months post-immunisation, RPS= 13.3%, which was not significantly different from control fish (chi-square; $P< 0.05$). No bacterial fish pathogens were detected in survivors of the groups of fish sampled at 14 days post-challenge.

Relative per cent survival of passively and actively immunised fish challenged with the virulent *V. anguillarum* 1, 2, 3 months post-immunisation is shown in Figure 3.6.

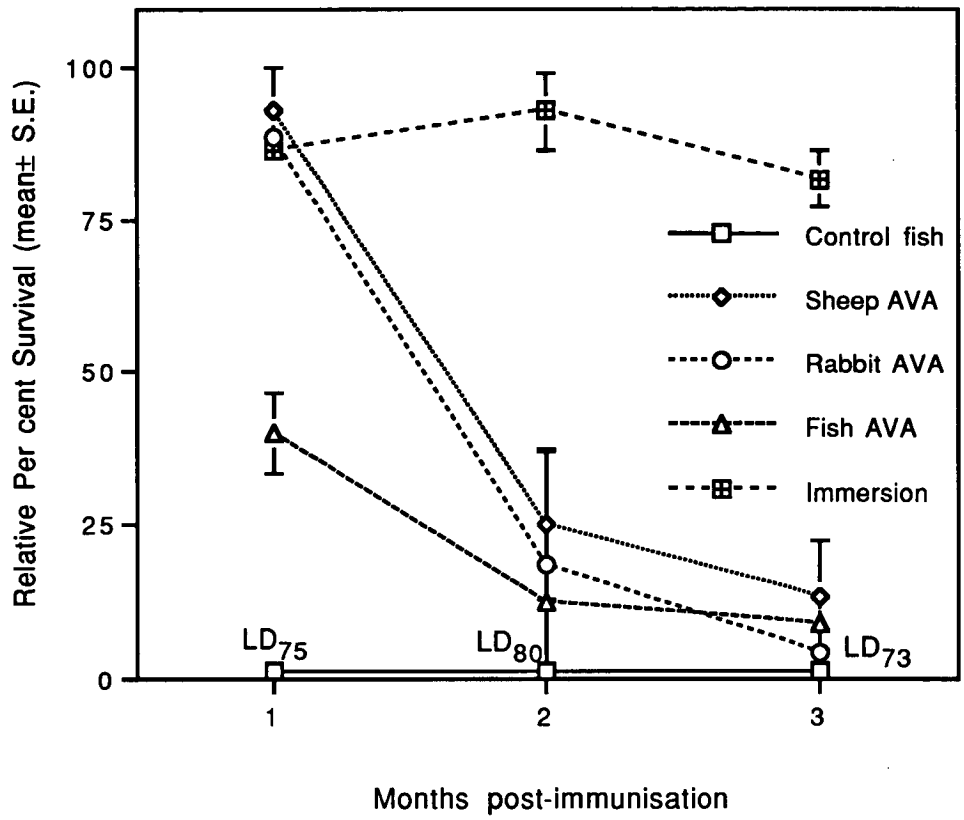


Figure 3.6: Relative per cent survival of passively and actively immunised fish challenged with the virulent *V. anguillarum* 1, 2, 3 months post-immunisation

3.8.4 **Discussion**:

Sheep AVA conferred high protection to fish which was not significantly different from that of immersion vaccination. It seems that the level and activity of immunoglobulins in 0.1 ml of antisera 100 g^{-1} body weight of fish was high enough to protect fish for at least one month after immunisation. These Ig are presumably distributed and stored in various organs following immunisation of fish.

The reason for the higher protection rate following administration of sheep AVA than those conferred by rabbit and fish AVA may be due to; 1) higher ELISA O.D (higher antibody level). 2) higher antibody functional activity against *Vibrio anguillarum*. 3) lower response by fish to sheep AVA resulting in greater persistence.

The lower RPS obtained from rabbit AVA is possibly due to the fish response to rabbit AVA (section 3.3.3). Nevertheless, both antisera significantly protected fish after one month post-immunisation ($P< 0.05$).

Fish AVA provided an RPS of 40% for rainbow trout challenged one month post-immunisation in this experiment (Table 3.6.2). This is in contrast with the findings of Harrell *et al.* (1975) who reported 90-95% protection 62 days post-immunisation following the injection of juvenile rainbow with 0.5 ml of rainbow trout

AVA in a challenge with 2.5x 10^5 *V. anguillarum*. However, 0.5 ml of the antisera was used in that study but 0.1 ml AVA $100g^{-1}$ fish body weight was only used in this experiment. Fish AVA did not protect rainbow trout significantly in challenges two and three months post-immunisation (Table 3.6.3 and 3.6.4).

These findings suggest that protective immunity was mainly due to specific Igs. However the collaboration of non-specific immunity was postulated as an essential factor to eliminate the infection. Investigations regarding the possibility of involvement of non-specific immunity in passive immunisation of fish is discussed later in this thesis (experiment VII).

The protection rate achieved with a single immersion vaccination was the same as protection conferred by i.p injection of sheep AVA. However, this protection was presumably conferred by cell mediated responses since no humoral antibody could be detected after one month post-vaccination. Also, immune memory may have played a role.

The mechanism of protection provided by sheep and rabbit antisera to *V. anguillarum* antigens presumably is simply in neutralising the biological effects of toxins and opsonising the bacteria. Furthermore, the level of protection and its duration in passively immunised fish will depend upon the rate at which the rabbit antibodies are complexed by the bacterial antigens present in the fish. Mammalian Ig would be mainly IgG, however, fish Ig are IgM possibly a more effective agglutinin.

Two month after immunisation, fish immunised passively with sheep, rabbit and fish AVA showed a moderate protection. This could be due to the high challenge rate (80% mortality) but was more likely due to the rate of clearance of these specific antibody to low levels after two months (section 3.2.3).

After three months of immunisation, the strongest protection occurred in fish actively immunised by immersion (Figure 3.6). This is consistent with the work of other researchers (Table 3.1, section 3.1.8) who reported a long protection following such a vaccination. At the time, those fish immunised passively with antisera were only marginally protected, if at all. This is consistent with the results of the rate of clearance of these antisera in the immunised fish after 70 days (section 3.2.3).

In all challenges, it was intended to keep the mortality rate at approximately 70% by using LD_{70} of live virulent bacteria as inocula. Nonetheless, slight variation due to time to time preparation of inocula affected the mortality pattern.

With respect to the horizontal transmission (section 2.11.1) during the challenges, it well reflects the more natural route of transmission of the disease for fish that are exposed and it express the protectiveness required by fish in order to escape natural infection. Only fish with a degree of immunity combat the disease. Thus, even a relatively low RPS from passive immunisation might be useful in the field where a mortality rate of 20% can easily occur by natural transmission.

3.7 Experiment VI Evaluation of protection against vibriosis using equivalent amounts of sheep, rabbit and fish anti-*Vibrio anguillarum* antibodies

3.7.1 Introduction and Aims:

This experiment was conducted in order to evaluate the protective value of the anti-*Vibrio* antibodies produced in sheep, rabbit and fish when they were given intraperitoneally in equivalent amount into fish. The aim was to evaluate how much sheep AVA is equivalent quantitatively to rabbit and fish AVA when given i.p. into fish fifteen and thirty days before experimental challenge. Equivalent binding activity in ELISA was initially used to standardise the dose of Ig.

3.7.2 Materials and Methods:

Antisera:

Anti-*Vibrio* antibodies (AVA) were produced as described in section 2.4. Fish were injected i.p. with 0.1 ml 100 g^{-1} their body weight of the antisera.

Fish:

Rainbow trout average weight 65± 8.22 g were held in two 4000 L temperature controlled tanks (15°C). After immunisation, fish were kept in the tanks until the time of challenge. The required number of fish from each group were taken and transferred to the isolation unit for experimental challenge at 15 days and the rest of fish left for 30 days post-immunisation (Table 3.7.1).

ELISA:

For the purposes of this experiment the ELISA reactivity of the different sera was used as the quantitative standard. Several ELISA were run to measure the activity of sheep, rabbit and fish AVA. Once the dilution at which the various ELISA gave the same reading were determined, the three kinds of antisera were diluted and the assay was run again to verify the procedure. The antisera for the immunisation trial were diluted just before intraperitoneal injection as follows:

First trial:

Sheep AVA: dilution of antisera to achieve O.D= 1.0.

5 µl Whole sheep AVA + 100 cc PBS (20,000 times dilution)

4.7 µl Ammonium sulphate purified (Amm. sul. pur.) sheep AVA + 100 ml PBS (21,000 times dilution).

100 µl Protein G purified sheep AVA + 70 ml PBS (700 times dilution).

Rabbit AVA: dilution of antisera to achieve O.D= 1.0.

100 µl Whole rabbit AVA + 20 ml PBS (200 times dilution)

100 µl Amm. sul. purified rabbit AVA + 20 ml PBS (200 times dilution).

100 µl Protein A purified rabbit AVA +30 ml PBS (300 times dilution).

Fish AVA: dilution of antisera to achieve O.D= 1.0.

Whole fish AVA (O.D= 1 measured by ELISA).

4 ml concentrated Amm. sul. pur.AVA + 16 ml PBS.(O.D= 1).

Concentrated amm. sul. pur.AVA (ELISA O.D= 1.4). This was not equivalent but was chosen to compare the higher degree of Ig content at this O.D at challenge.

Fish were injected with 0.1 ml 100 g body weight $^{-1}$ of antisera, each with an ELISA O.D= 1.

Immunised fish were challenged 15 and 30 days post-immunisation (Table 3.7.1).

Table 3.7.1: Experimental design for challenge of passively immunised fish with equivalent amounts of antisera

Treatment	Route of administration of antisera	Number of fish challenged (two replicates)	
		15 days	30 days
Whole sheep serum	i.p.	20	20
Ammonium sulphate purified sheep serum	i.p.	20	20
Protein G purified sheep AVA	i.p.	20	20
Whole rabbit serum	i.p.	20	20
Ammonium sulphate purified rabbit serum	i.p.	20	20
Protein A purified rabbit AVA	i.p.	20	20
Whole fish serum	i.p.	20	20
Ammonium sulphate precipitated fish serum	i.p.	20	20
1:4 diluted ammonium sulphate precipitated fish AVA	i.p.	20	20
Control Challenged non-immune	-	20	20
		Total No. of fish= 400	

AVA= Anti-*V. anguillarum* antisera

Dry weight of antisera:

In order to have an estimation of dry weight of the amount of anti-*Vibrio* antibodies in the immune sera, 1 ml of different non-immune and immune sera were freeze dried.

Quantitative precipitin test of sheep, rabbit and fish AVA:

In an attempt to measure the antibody levels in three different sera quantitatively, different concentrations of lipopolysaccharide (LPS) antigen were added to a constant amount of each antibody in series of tubes and the antibody content was measured as described by Heidelberger and Kendall (cited by Hudson and Hay, 1989). Immunodiffusion was also conducted to evaluate the quantitative level of antibodies.

LPS was prepared by phenol extraction method described by Westphal and Jann (1965). Briefly, *V. anguillarum* cells , after cultivation in vibrio broth for 24 h, were centrifuged, and the sediment was washed with PBS. The bacteria were then lyophilised and twenty gram (dry weight) of bacteria were suspended in 350 ml of distilled water at 68°C (on a water bath); 350 ml of 90% phenol, preheated to 68°C, was then added with vigorous stirring and the mixture was kept at 68°C for 15 min . After cooling to about 10°C by placing the vessel in an ice bath, the emulsion was centrifuged at 3000 r.p.m for 45 min, which resulted in the formation of three layers. The water phase was collected and dialysed for 3 days against distilled water to remove phenol and small amounts of low-molecular-weight bacterial substances. The resulting product contained 40-50% of lipopolysaccharide and 50-67% of bacterial ribonucleic acid. To eliminate the bacterial ribonucleic acid, the extract was centrifuged for 8 h at 80,000 x g. The sediment was then suspended in distilled water and the suspension was recentrifuged 3 times at 105,000 x g for 3 h. The final sediment was taken up in a minimum amount of water and freeze-dried. The yield of bacterial lipopolysaccharide was 420 mg.

Radial immunodiffusion:

This test was performed to measure the antibody level of sheep, rabbit and fish AVA. Briefly, sheep, rabbit or fish AVA was incorporated into three flasks of agar (1 ml of AVA into 100 ml 45-50°C agar). Plates were left to solidify and then 25 µl of antigen [LPS (5 mg ml^{-1})] was placed in several wells into agar. Plates were incubated overnight in a moist chamber at room temperature. After 24-48 h the area of precipitation ring was measured and compared with different sera in different plates. This test was repeated 5 times with 0.1, 0.2, 0.4, 0.6 and 0.8 ml of the AVA incorporated into agar against concentrations of 20, 15, 10, 5 and 2 µl of the LPS respectively.

Complement fixation test:

Complement fixation test (CFT) utilising LPS was also performed to evaluate the antibody activity in sheep, rabbit and fish AVA. Briefly, the antigen (5 mg ml^{-1}) and the antiserum (dilution 1: 40) under test (deprived of its complement by heating at 56°C for sheep and rabbit and 44°C for fish) were incubated in the presence of guinea-pig serum, which provided a source of complement. After allowing the antigen-antibody-complement mixture to react for 20 min at 37°C, antibody-coated sheep red blood cells (RBC) was added. Microplate was incubated for 20 min at 37°C and centrifuged. Lysis of the RBC was a negative result indicated that the complement was not fixed. Absence of lysis of RBC [(no lysis (4+) to 50% lysis (1+) dependent upon the amount of complement unbound)] was a positive result indicated that the antigen reacted with the specific antibody and then complement bound to the immune complex. Before performing the CFT, all reagents including complement, sheep RBC, antibody against RBC (haemolysin), antigen and test sera were optimised

Virulent *V. anguillarum* for challenge:

Challenge was conducted at 15 days after immunisation on half of the immunised fish. Fish were challenged with virulent bacteria as described in section 2.10.1. In this experimental challenge, fish were inoculated with 2.2x 10^7 cfu 0.1 ml^{-1} ($LD_{84.21}$), and 2.1x 10^7 cfu 0.1 ml^{-1} (LD_{85}) of live *V. anguillarum* cells for 15 days, 30 days challenges respectively.

3.7.3 **Results:**

Table 3.7.2 shows the mortality and RPS after challenge of the passively immunised fish with equivalent amounts of antisera (ELISA O.D= 1) fifteen days post-immunisation.

Trial 1

Table 3.7.2: Mortality and RPS after challenge (with virulent *V. anguillarum*, $LD_{84.21}$) of rainbow trout passively immunised with equivalent (measured by ELISA O.D= 1.0) sheep, rabbit and fish AVA at 15 days p.i. as compared to controls

Treatment	Number of mortality caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
Whole sheep serum@	8/10	7/10	15/20	11.7 ± 5.88
Amm. sul precipitated Sheep serum@	7/10	8/10	15/20	11.7 ± 5.88
Protein G Purified sheep serum@	4/10	4/10	8/20	53.0 ± 0.0
Whole Rabbit serum@	5/10	7/10	12/20	29.4 ± 11.76
Amm. sul precipitated rabbit serum@	7/10	7/10	14/20	17.6 ± 0.0
Protein-A purified rabbit serum@	7/10	6/10	13/20	23.5 ± 5.88
Whole fish AVA	1/10	1/10	2/20	88.2 ± 0.0
Amm. sul. precipitated fish AVA	1/10	0/10	1/20	94.1 ± 5.88
1:4 diluted Amm. sul. precipitated fish AVA	2/10	3/10	5/20	70.6 ± 5.88
Control Challenged non-immune	8/10	9/10	17/20	0.0 ± 5.88

AVA (Anti-*Vibrio anguillarum*) Amm. sul. = Ammonium sulphate

* Experimental challenge LD_{85}= $2.2x10^7$ cfu 0.1^{-1} ml by i.p injection.

@ Anti-V. anguillarum antisera adjusted to ELISA O.D of fish AVA (O.D= 1.0)

Despite the equal ELISA activity of the injected AVA (ELISA O.D= 1.0), there was not a significant difference (P< 0.05) between groups of fish immunised with equivalent sheep and rabbit compared to control fish. The protection obtained was irrelevant to ELISA activity of these sera. Protein-G purified sheep AVA protected fish with a higher survival rate (RPS of 53.0%), although the ELISA activity of these sera was O.D= 1.0. None of the rabbit AVA (O.D= 1.0) could protect fish against vibriosis.

Whole fish AVA and ammonium precipitated fish AVA equally protected fish in the challenge at 15 days post-immunisation. Protections obtained from immunisation of fish with whole fish serum (RPS of 88.2%) and ammonium sulphate precipitated fish serum (RPS of 94.1%) were not significantly different from each other (P< 0.05). Protections obtained in the challenge after 15 days post-immunisation in the fish immunised with equivalent amounts of antisera (O.D= 1.0) are also shown in Figure 3.7.1.

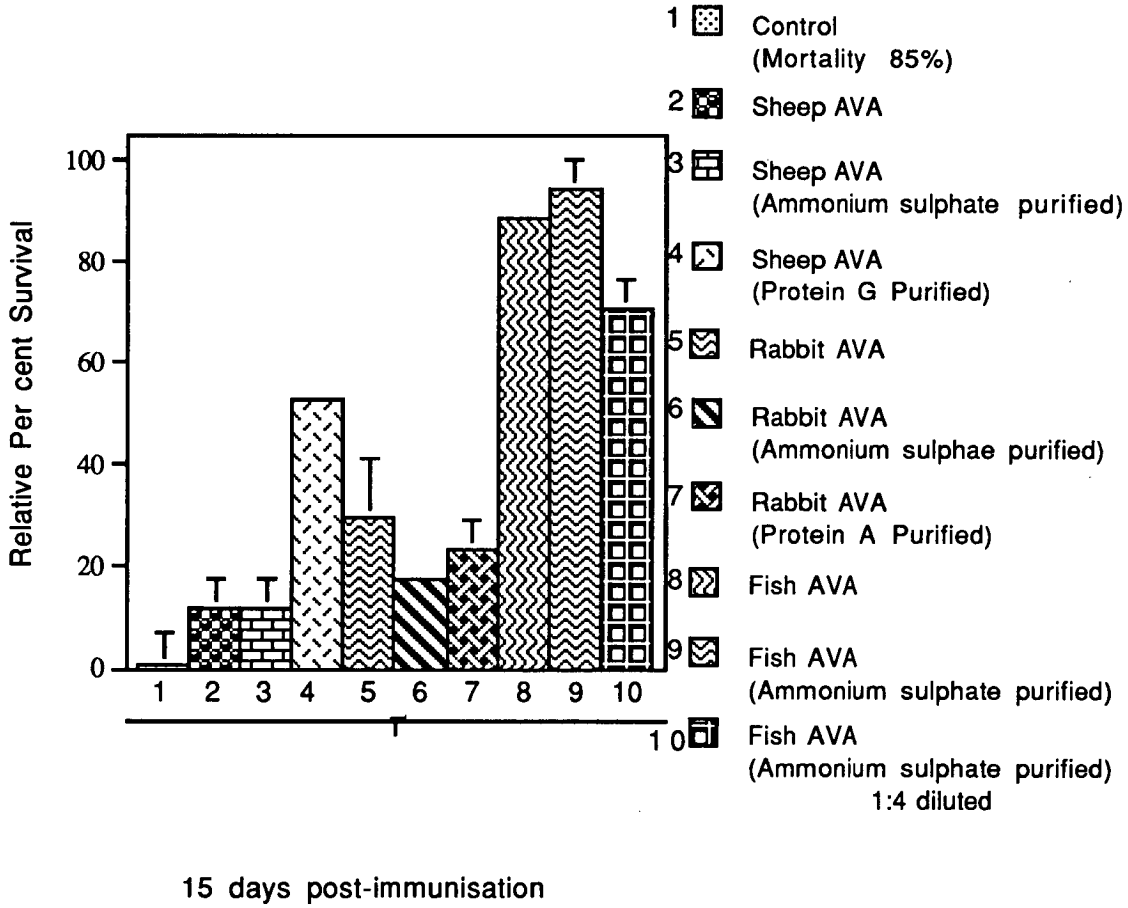


Figure 3.7.1: Protection rate of equivalent amounts (ELISA O.D= 1.0) of sheep, rabbit and fish AVA

Trial 2 (modifying the experimental design):

Since no protection was observed after 15 days of immunisation on half of the immunised fish in groups of fish immunised with equivalent sheep and rabbit diluted and adjusted to an equivalent O.D to that of fish (measured by ELISA) in trial 1, the

remaining fish except those immunised with fish AVA antisera (kept for a 30 days challenge, results in Table 3.7.5) were divided in two 6 groups (6 groups for sheep AVA and 6 groups for rabbit AVA injection, each group consisted of 10 fish) in such a way that partially immunised fish receive the highest amount of AVA (low dilution of AVA) and injected with different dilutions of sheep and rabbit AVA as follows.

Dilution of sera:

Sheep AVA injected fish:

1: 5 dilution; 1 ml whole sheep AVA + 4 ml PBS (5 times dilution)
1: 50 dilution; 0.1 ml whole sheep AVA + 4.9 ml PBS (50 times dilution).
1: 250 dilution; 20 µl whole sheep AVA + 4.98 ml PBS (250 times dilution).
1: 750 dilution; 10 µl whole sheep AVA + 7.49 ml PBS (750 times dilution).
1: 2500 dilution; 2 µl whole sheep AVA + 4.99 ml PBS (2500 times dilution).
1: 5500 dilution; 2 µl whole sheep AVA + 10.98 ml PBS (5500 times dilution).

Rabbit AVA injected fish:

1: 2 dilution; 2.5 ml rabbit AVA + 2.5 ml PBS (2 times dilution)
1: 4 dilution; 1 ml rabbit AVA + 4 ml PBS (5 times dilution)
1: 8 dilution; 1 ml rabbit AVA + 7 ml PBS (8 times dilution)
1: 20 dilution; 250 µl rabbit AVA + 4.75 ml PBS (20 times dilution)
1: 30 dilution; 200 µl rabbit AVA + 5.8 ml PBS (30 times dilution)
1: 60 dilution; 100 µl rabbit AVA + 5.9 ml PBS (60 times dilution)

Each group consisted of only one replicate since previous results showed a relatively small variation between replicates. Furthermore, there was not enough fish available with the same size of the experimental fish for setting up a replicate. The results of challenge of immunised rainbow trout with diluted sheep AVA and diluted rabbit AVA are shown in Table 3.7.3 and Table 3.7.4 respectively.

Table 3.7.3: Mortality and RPS after challenge (with virulent *V. anguillarum*, LD_{85}) of rainbow trout passively immunised with different quantities of sheep AVA at 15 days p.i. as compared to controls

Treatment	Number of mortality caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates.	RPS± S.E.
Sheep AVA (1:5 dilution)	0/10	-	-	100
Sheep AVA (1:50 dilution)	2/10	-	-	76.4
Sheep AVA (1:250 dilution)	8/9	-	-	0
Sheep AVA (1:750 dilution)	8/10	-	-	5.88
Sheep AVA (1:2500 dilution)	9/10	-	-	0
Sheep AVA (1:5500 dilution)	9/9	-	-	0
Control Challenged non-immune	8/10	9/10	17/20	0.0 ±5.88

* Experimental challenge LD_{85}= $2.1x10^7$ cfu 0.1^{-1} ml by i.p. injection.

Table 3.7.4: Mortality and RPS after challenge (with virulent *V. anguillarum*, LD_{85}) of rainbow trout passively immunised with different quantities of rabbit AVA at 15 days p.i. as compared to controls

Treatment	Number of mortality caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
Rabbit AVA (1: 2 dilution)	1/10	-	-	88.2
Rabbit AVA (1: 4 dilution)	1/10	-	-	88.2
Rabbit AVA (1: 8 dilution)	2/9	-	-	73.8
Rabbit AVA (1: 20 dilution)	4/10	-	-	52.9
Rabbit AVA (1: 30 dilution)	3/10	-	-	64.7
Rabbit AVA (1: 60 dilution)	5/10	-	-	41.1
Control Challenged non-immune	8/10	9/10	17/20	0.0 ±5.88

* Experimental challenge LD_{85}= $2.1x10^7$ cfu 0.1 ml^{-1} i.p injection.

It can be seen from the results that the equivalent quantity of three different antiserum which can confer immunity to fish against experimental infection is as follows:

1:50 dilution of sheep AVA ~ 1:8 dilution rabbit AVA ~ Whole fish AVA

Table 3.7.5 shows the mortality and relative per cent survival of rainbow trout immunised with fish AVA (ELISA O.D= 1.0) and challenged 30 days post-immunisation.

Table 3.7.5: Mortality and RPS after challenge (with virulent *V. anguillarum*, LD_{85}) of rainbow trout passively immunised with different quantities of fish AVA at 30 days p.i. as compared to controls

Treatment	Number of mortality caused by *V.anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates.	RPS± S.E.
Whole fish AVA	7/10	7/10	14/20	17.6 ± 0.0
Amm.sul. precipitated fish AVA	6/10	5/10	11/20	35.2 ± 5.88
1:4 diluted amm. sul. precipitated Fish AVA	7/10	8/10	15/20	11.7 ± 5.89
Control Challenged non-immune	8/10	9/10	17/19	0.0 ± 5.88

* Experimental challenge $LD_{85}=2.1x10^7$ cfu/ 0.1 ml i.p injection.

Obviously the fish ammonium sulphate precipitated antiserum provided fish with more AVA antibodies. Therefore, the fish resisted the infection with higher RPS (RPS= 35.29%). However, RPS dropped when this serum was diluted 1:4 (RPS= 11.7%).

Dry weight of antisera:

Table 3.7.6 shows the dry weight of 1 ml of different immune AVA and non-immune sheep, rabbit and fish sera.

Table 3.7.6: Dry weight mg ml^{-1} of whole non-immune and immune anti-*V. anguillarum* antiserum of fish, rabbit and sheep measured after freeze drying

Serum of	Non- immune sera			Immune sera		
	Whole serum	Amm.sul. precipitated	Protein A / G purified	Whole serum	Amm.sul. precipitated	Protein A / G purified
Fish	23	10	-	40	25	-
Rabbit	29	25	10	48	36	14
Sheep	50	16	5	148	86	9

Amm.sul.= Ammonium sulphate

Quantitative precipitation using *V. anguillarum* LPS:

Results of quantitative precipitation using *V. anguillarum* LPS test showed more precipitation with rabbit AVA than sheep and fish AVA. Rabbit antiserum had 4.12 mg ml^{-1} and sheep AVA had 3.98 mg ml^{-1} precipitin antibody content. However, fish AVA did not precipitate with LPS (Figure 3.7.2).

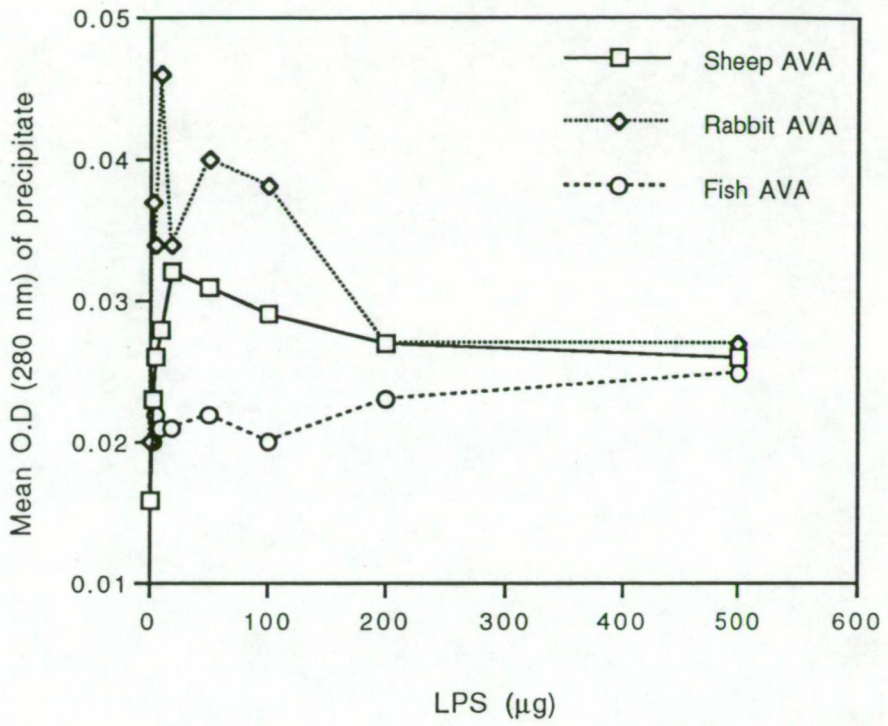


Figure 3.7.2: Optical density of precipitate of sheep, rabbit and fish AVA vs different concentrations of *V. anguillarum* LPS (µg)

Radial immunodiffusion:

The results of radial immunodiffusion measuring the antibody level of sheep, rabbit and fish against *V. anguillarum* using sonicated *V. anguillarum* and the prepared LPS as antigen, showed that rabbit AVA have more precipitin activity than sheep AVA while fish AVA did not appear to show any precipitin activity.

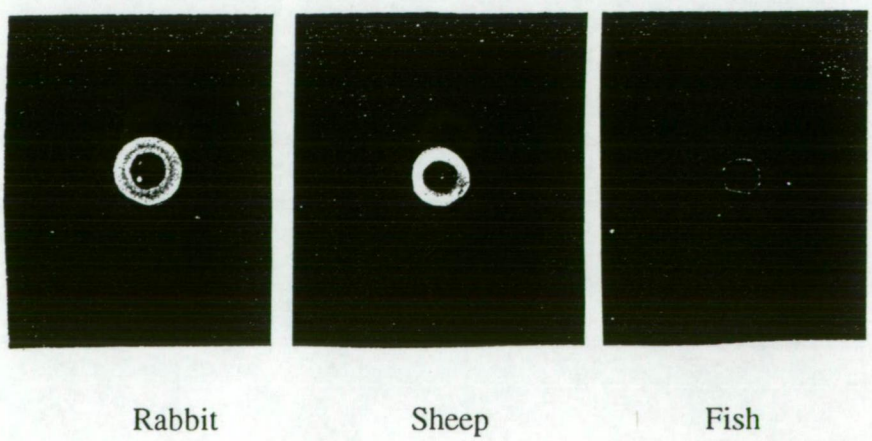


Plate 3.7: Radial immunodiffusion of rabbit, sheep and fish AVA against *V. anguillarum* LPS

Complement fixation test:

Complement fixation test results utilising LPS as antigen with whole sheep, rabbit and fish AVA showed that rabbit antibodies fixed the complement more strongly (4+) than sheep antibodies (2+) whereas fish antibodies (-) did not show any complement fixing reactivity.

3.7.4 **Discussion:**

The equivalence activity of antisera *in vitro* measured by ELISA was not the same as *in vivo* when they were used in protection trials. The activity of each antiserum i.e sheep AVA was measured by sheep ELISA system where the type, affinity and avidity of immunoglobulins as well as the type of conjugate as a secondary antibody were all different from the rabbit ELISA system. Also, in the fish ELISA system a monoclonal antibody and an extra anti-secondary antibody was used. Therefore, the variation between the ELISA and protection results was predictable.

Protein-G purified sheep conferred significantly higher protection ($P<0.05$) than whole and ammonium sulphate precipitated sheep AVA (with the same ELISA O.D) (Table 3.7.2). It is obvious that the level of antibodies in the purified sera was greater than the whole sera even though the ELISA O.D of these sera were the same. It is difficult to make a conclusion while the ELISA O.Ds of these three sera were the same, the rate of protection they conferred to fish was different.

Regarding the results of trial 2, a dilution of 1: 50 of sheep AVA was equivalent to the dilution of 1: 8 of rabbit and both of sheep and rabbit AVA in these dilutions provided fish with the same protection resulted from injection of immunised fish AVA into non-immune fish in the challenge 15 days after immunisation. It can be concluded that sheep anti-*Vibrio* immunoglobulins are 50 times and rabbit anti-*Vibrio* immunoglobulins are 8 times either quantitatively or qualitatively more potent than fish immunoglobulins in protecting fish from the infection in spite of the fact that their dry weight measured by freeze drying (Table 3.7.6) were not much far quantitatively. However, if the quantity is considered, rabbit AVA showed more precipitin antibodies. Therefore, the difference might be due to quantity of antigen-antibody of rabbit AVA and lack of other properties in neutralising bacterial antigens when compare with sheep AVA.

By subtracting non-immune dry weights from immune dry weights of sheep and rabbit sera (Table 3.7.6), the differences are presumably due to anti-*Vibrio* antibodies produced as a result of immunisation (Table 3.7.7). When this difference is divided by the serum dilution factor required for equivalent protection of fish (1: 50 for fish sera, 1; 8 for rabbit sera), the result can be considered to represent units of antibody activity. For sheep and rabbit the weights of Ig required for equivalent protection were similar (Table 3.7.7).

Table 3.7.7: Correlation of dry weight (mg ml^{-1}) with the protection efficacy of rabbit and fish AVA

Sera of	Immune, non-immune (whole serum)	Immune, non-immune (amm.sul. precipitated)
Rabbit	19 mg	11 mg
	19/ 8*= 2.38 mg	11/ 8*= 1.37 mg
Sheep	98 mg	70 mg
	98/ 50*= 1.95 mg	70/ 50*= 1.4 mg
Fish	17 mg	15 mg
	17/ 1*= 17 mg	15/ 1*= 15 mg

* Ratios were obtained from the protection experiment (trial 2)

This does not work similarly with fish presumably due to the fact that fish Ig are IgM like with a high molecular weight and those of mammalian are mainly IgG with lower molecular weight. It is also possible that other proteins rather than Ig are precipitated and constitute some weights. Therefore, further work is required to elucidate this.

Rabbit AVA showed higher precipitating antibody than sheep with an equivalent antigen (LPS) amount (Figure 3.7.2). This is probably due to higher precipitin activity of rabbit Ig. It is in contrast with what obtained *in vivo* where sheep AVA was superior to rabbit AVA. Fish on the other hand did not show precipitin in either of the tests suggesting presumable lack of precipitin activity. Fish IgM does not possibly diffuse in gels and even when incorporated in gel as sheep and rabbit IgG do. Therefore, precipitin evaluation is probably useful for comparing within species but not inter- species. Moreover, the method used for equivalence of antigen- antibody is well defined for soluble antigen such as human serum albumin and antibodies such as anti-human albumin, but not for bacterial soluble antigen and the corresponding antibody response. Agglutinating antibodies also was not appropriate in comparing antibody equivalence of three different species (section 2.8). Normal fish serum was not used in complement fixation test for fish because the aim was to compare sheep,

rabbit and fish sera activity in this test. Therefore, it is possible that immune complexes in fish CFT could not fix guinea pig complement.

Thus, according to the results obtained from protection trial in this experiment, in order to obtain an equivalent protection, fish, rabbit and sheep antisera required to be diluted 1: 8 and 1: 50 respectively. However, these dilution levels were not coincident with those required to give equivalent ELISA O.D readings which were 1: 200 and 1: 20,000 for rabbit and sheep sera respectively. Consequently, while ELISA titres provided valuable information on the relative potencies of antisera, the *in vitro* systems used in these observations did not permit direct biological comparisons.

Regarding multiple injections of fish in the second challenge and the possibility of any conferred protection, it is clearly shown (in the non-specific trial, section 3.8) that injection itself or with a non-immune serum could not confer protection.

3.8 Experiment VII Evaluation of non-specific protective factors associated with passive immunisation

3.8.1 Introduction and Aims:

Fish depend more on their non-specific defense mechanisms than the specific immune response for protection against diseases (Anderson, 1992). It has been shown from different protection trials that fish may respond non-specifically when injected with a variety of products even, non-immune serum. Examples of this are as follows:

Intraperitoneal injection of either sterile or formalin-inactivated *Aeromonas salmonicida* caused an increase in serum lysozyme in rainbow trout. It was suggested that a number of antibacterial factors increased in concentration following immunisation and that these probably played a role in protection against microbial disease (Rainger and Rowley, 1993).

Murai *et al.* (1990) showed that a C-reactive protein (CRP) in the serum of rainbow trout increased in concentration following immunisation with *V. anguillarum* and it was suggested that this glycoprotein was important in defense against bacterial infection.

Ellis *et al.* (1988) reported that control rabbit serum (rabbit injected with Freund's complete adjuvant alone) injected into fish resulted in some degree of protection compared with saline-injected fish; the reason for this was unknown. Control rabbit serum possessed no detectable antibodies to *A. salmonicida* antigens in any of the assays used. The protective effect was, therefore, probably due to non-specific effects possibly relating to toxin neutralisation or macrophage activation via, for example, interleukins (Ellis *et al.*, 1988). Also, there were fewer mortalities in groups of Atlantic salmon injected with control serum (non-immune rainbow trout serum) than in the non-injected control when Atlantic salmon were challenged with virulent *A. salmonicida* (Cipriano, 1982).

The aim of this study was to determine if serum complement components in non-immune and immune whole serum from sheep, rabbit and fish contained substances capable of providing non-specific protection or stimulating non-specific responses of fish.

3.8.2 Materials and Methods:

Antisera: Antisera were the same as described in section 2.4. For complement inactivation of fish serum a heat treatment at 44°C for 20 min was used (Sakai, 1981a). For rabbit and sheep sera 56°C heat for 30 min was used (Sakai, 1981b). Complement inactivation of sera was carried out immediately before their use.

Fish: Rainbow trout average weight 120± 11 g kept in temperature controlled tanks (15°C). Twenty fish were used for each treatment in two replicates (Table 3.8.1). Fish were injected with 0.1 ml 100 $g^{-1}$of the antisera.

Virulent *V. anguillarum* with LD_{70} for challenge:

Half of fish were allocated for challenge at 15 days and the other half for 30 days challenge. Fish were challenged with the virulent bacteria as described in section 2.10.1. In this experimental challenge, fish were inoculated with 1.2×10^7 cfu 0.1 ml^{-1} ($LD_{84.21}$), and 7.2×10^7 cfu 0.1 ml^{-1} (LD_{90}) of live *V. anguillarum* cells at 15 and 30 days challenges respectively.

Table 3.8.1: Experimental design for challenge of fish received non-immune and immune sera to assess the effect of complement

Treatment	Route of administration of antisera	Number of fish challenged(in two replicates)	
		15 days	30 days
Non-immune sheep serum	i.p.	20	20
Heated++ non-immune sheep serum	i.p.	20	20
Heated++ immune sheep AVA	i.p.	20	20
Non-immune rabbit serum	i.p.	20	20
Heated++ non-immune rabbit serum	i.p.	20	20
Heated++ immune rabbit AVA	i.p.	20	20
Non-immune fish serum	i.p.	20	20
Heated++ non-immune fish serum	i.p.	20	20
Heated++ immune fish AVA	i.p.	20	20
Control Challenged non-immune	-	20	20
			Total No. of fish= 400

AVA= Anti *Vibrio anguillarum* antisera

3.8.3 **Results:**

Fifteen-day challenge:

Protection obtained from i.p. injected fish by immune and non-immune, with complement or without complement in a challenge ($LD_{84.21}$) after 15 days post-immunisation is shown in Table 3.8.2. Only the immune sera provided protection fifteen days after immunisation.

Table 3.8.2: Mortality and RPS after challenge (with virulent *V. anguillarum*, $LD_{84.21}$) of rainbow trout passively immunised with non-immune, heated AVA and non-immune heated sheep, rabbit, and fish at 15 days p.i. as compared to controls

Treatment	Number of mortalities caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
Non-immune sheep serum	9/10	8/10	17/20	0.0 ± 5.93
Heated++ immune sheep AVA	0/10	0/10	0/20	100 ± 0.0
Heated++ non-immune sheep serum	8/10	9/10	17/20	0.0 ± 5.93
Non-immune rabbit serum	7/10	8/10	15/20	1.1 ± 4.44
Heated++ immune rabbit AVA	0/10	0/10	0/20	100 ± 0.0
Heated++ non-immune rabbit serum	9/10	9/10	18/20	0.0 ± 0.0
Non-immune fish serum	8/10	9/10	17/20	0.0 ± 5.93
Heated++ immune fish AVA	0/10	1/10	1/20	94.1 ± 5.94
Heated++ non-immune fish serum	7/10	9/10	16/20	5.0 ± 11.87
Control Challenged non-immune	8/10	8/9	16/19	0.0 ± 5.26

* Experimental challenge $LD_{84.21} = 1.2 \times 10^7$ cfu 0.1 ml^{-1} by i.p. injection.

A complete protection was observed following the injection of 0.1 ml 100 g^{-1} fish body weight of the complement inactivated sheep and rabbit and fish AVA.

Protection conferred by fish AVA markedly protected fish after 15 days post-immunisation (RPS= 94.1%) and this was not significantly different ($P< 0.05$) from the protection conferred by sheep and rabbit AVA.

Thirty-day challenge:

After 30 days post immunisation, complete protection was observed following the injection of 0.1 ml 100 g^{-1} fish body weight of complement inactivated sheep AVA. A RPS of 83.3% was obtained for rabbit AVA that was not significantly different ($P< 0.05$) from the protection conferred by sheep AVA (RPS of 100%). Fish AVA marginally protected fish (RPS= 16.66%) after 30 day of passive immunisation which was not significantly different from control fish ($P< 0.05$). No protection was observed following the injection of non-immune sera (Table 3.8.3).

Table 3.8.3: Mortality and RPS after challenge (with virulent *V. anguillarum* , LD_{90}) of rainbow trout passively immunised with non-immune, heated AVA and non-immune heated sheep, rabbit, and fish at 30 days p.i. as compared to controls

Treatment	Number of mortalities caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
Non-immune sheep serum	8/10	9/10	17/20	5.5 ± 5.55
Heated++ immune sheep AVA	0/10	0/10	0/20	100 ± 0.0
Heated++ non-immune sheep serum	9/10	9/10	18/20	0.0 ± 0.0
Non-immune rabbit serum	9/10	10/10	19/20	0.0 ± 5.55
Heated++ immune rabbit AVA	1/10	2/10	3/20	83.3 ± 5.55
Heated++ Non-immune rabbit serum	9/10	9/10	18/20	0.0 ± 0.0
Non-immune fish serum	10/10	9/10	19/20	0.0 ± 5.55
Heated++ immune fish AVA	8/10	7/10	15/20	16.6 ± 5.56
Heated++ non-immune fish serum	10/10	10/10	20/20	0.0 ± 0.0
Control Challenged non-immune	10/10	8/10	18/20	0.0 ± 11.10

* Experimental challenge LD_{90}= 7.2×10^7 cfu 0.1 ml^{-1} by i.p. injection

The results of relative per cent survival obtained following challenge of immunised fish with immune and non-immune, complement inactivated or normal sera are also shown in figure 3.8.

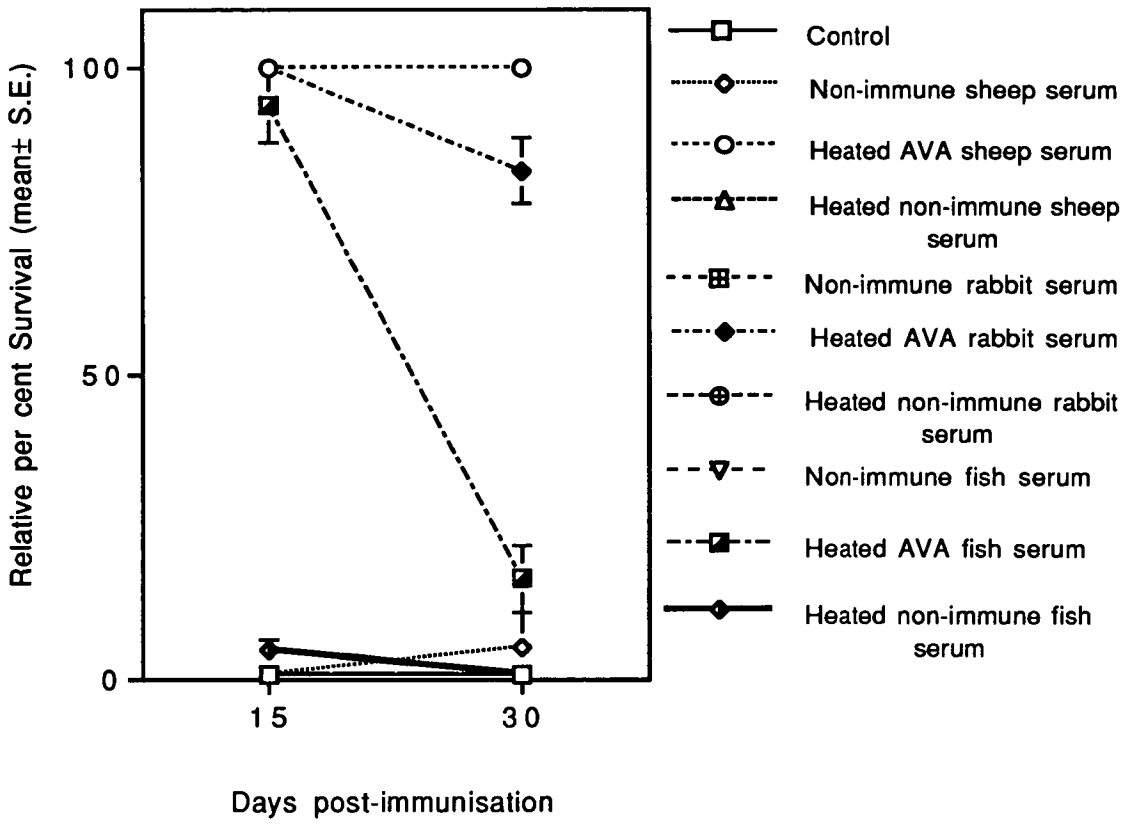


Figure 3.8: Protection obtained from complement inactivated and normal immune (AVA) and non-immune sera

3.8.4 **Discussion:**

The results showed that the protection was not related to complement and was therefore almost certainly due to antibodies in the serum. Complement inactivated sera and sera with complement protected fish equally. It is probable that by heating the sera in the certain temperatures used, some components of complement were not inactivated. Thus, the similarity of complement-inactivated sera and sera with complement might be due to the existence of some complement components. Further research is required to elucidate this.

The non-immune sera used in this experiment were the sera of non-immune animals that did not confer protection against vibriosis to fish. However, Ellis *et al.* (1988) found that control rabbit serum (rabbit injected with Freund's complete adjuvant alone) resulted in some degree of protection against furunculosis compared with saline-injected fish. It is well known that mycobacterial antigens can stimulate a range of non-specific immune factors. Therefore, the rabbit serum could have conferred some degree of protection to fish. Also, it is not understood why the trial challenge using non-immune rainbow trout serum against *A. salmonicida* gave marginal protection to brook trout (Cipriano, 1982). It is possible, however, that the

pattern of non-specific responses of fish to furunculosis may also be different from that of vibriosis.

The complement inactivated sheep, rabbit or fish AVA were as protective (in this experiment) as the unheated sera (experiment V). This is in agreement with Olivier *et al.* (1985) who found complement inactivated rabbit anti-*Aeromonas salmonicida* (RAS) was as protective as unheated RAS.

RPS of 5 and 5.5% obtained due to injection of fish with non-immune sera is probably due to individual differences between fish, however, they were not significantly different from control ($P< 0.05$).

The differences observed between the rate of protection of rabbit AVA and fish AVA after 15 days and one month post-immunisation in this trial compared with previous trials (section 3.2) are presumably due to the different nutrition and condition variation of individual fish used.

The other component of serum such as lysozyme did not appear to confer any protection against vibriosis since they were included in the inoculum (non-immune serum). However, the lysozyme content of non-immune sera was not determined before their use.

These observations suggest that protective immunity was mainly due to the activity of specific Ig. Protection appears to be associated with specific antibodies and not complement at least in the case of immune rabbit serum against *A. salmonicida* (Olivier *et al.*, 1985). However the collaboration of non-specific immunity probably has been an essential factor to eliminate the infection. Further work is required to elucidate the role of non-specific immunity in fish.

3.9 Experiment VIII Passive immunisation by the oral route using the carriers, Quil-A, Iscar and LTB .

3.9.1 Introduction:

Oral delivery of vaccines has long been regarded as an ideal method for fish farmers to control diseases. Nonetheless, it has been difficult for scientist to make it applicable as an effective route for the aquaculture industry. Although immersion and intraperitoneal vaccination methods have proved beneficial in the industry, oral immunisation is still very restricted in its use (Hart *et al.*, 1988; Lillehaug, 1989).

Georgopoulou *et al.* (1985, 1986) showed that the epithelial cells of the posterior intestine in both young and adult trout are structurally and functionally comparable to those of the ileum of the suckling rat that play a basic nutritional role and proteins which do not undergo hydrolysis in the lumen or protein fragments, pass into the large vacuolar system characteristic of these cells, where they are digested. The intestinal tract of teleost fish is regionally differentiated, the anterior intestine are associated with lipid absorption and the posterior intestine can carry out macromolecule endocytosis (Georgopoulou *et al.*, 1988).

Evidence has shown that proteins were detected in fish blood plasma and various tissues when they were administered orally to the fish, for example, plasma and tissue accumulation of HRP (McLean and Ash, 1987) and HRP with the antiprotease, soybean trypsin inhibitor and a synthetic detergent following oral delivery to rainbow trout (McLean and Ash, 1990). Orally administered HRP crossed the intestinal epithelium into the circulation and there was a direct correlation between the quantity ingested and the quantity transferred to the plasma within the experimental limits chosen (Georgopoulou *et al.*, 1988). Transport of orally administered rabbit Ig into blood circulation of goldfish (*Carassius auratus*) has also been shown (Nakamura *et al.*, 1990).

Tests on plasma from coho salmon vaccinated orally with *V. ordalii* lipopolysaccharide revealed antibodies against *V. ordalii* LPS and passive transfer of this plasma to naive coho also resulted in measurable protection of the recipient coho against *V. ordalii* (Velji *et al.*, 1993).

Several novel delivery systems have been employed to carry antigens through fish gut e.g. the saponin adjuvant Quil-A as a direct adjuvant had a direct effect on the intestine of the tilapia in regard to the level of absorption of human gamma globulin and subsequent cellular localisation (Jenkins *et al.*, 1991).

Aizpurua and Russell-Jones (1988) showed that oral administration of LTB (the GM-1-binding subunit of *Escherichia coli* heat-labile toxin) to mice elicited significant antibody titres. It was proposed from this work that LTB is able to bind to glycolipids and glycoproteins on the intestinal mucosa and stimulate these cells to transport the protein into the systemic circulation, thereby eliciting a systemic immune response.

Immunisation of rabbit and mice injected with bovine serum albumin coupled to TraT (immuno-stimulatory carriers, an internal membrane of *E. coli*) resulted in the production of high titres of antibody without need for oil-based adjuvants (Croft *et al.*, 1991). TraT has been shown as a T-cell immunopotentiating agent (Bell Stephen *et al.*, 1993).

These results indicate the need for further research to evaluate oral delivery of proteins either as immunoglobulins for passive immunisation or microbial extracts for active immunisation purposes.

The main aim of this experiment was to evaluate the protective value of purified sheep anti-*Vibrio* antibodies (AVA) when they were conjugated to some known protein carriers like Quil-A, TraT and LTB and fed to rainbow trout.

3.9.2 **Materials and Methods:**

Purified anti-*Vibrio anguillarum* (AVA):

Purification of sheep antisera (sheep AVA) using protein-G was carried out as described in section 2.3. Briefly fractions of 5 ml were collected and immediately neutralised by appropriate µl of 2M Tris, pooled and extensively dialysed against multiple changes of Tris saline for 24 h at 4°C, pooled, concentrated against Aquacide II, aliquoted and stored at -20°C. The yield of affinity purified Igs from pools were 9.4 mg/ml after dialysis against, and concentrated using, Aquacide II. ELISA activity of the purified AVA was 1.8 (O.D= 405 nm) at a dilution of 1: 200.

Carriers:

Quil-A (Superfos Biosector a/s, Denmark) 0.03% solution, TraT and LTB (Biotech Australia); TraT 50 µg/fish and LTB 100 µg/fish were used as protein carriers.

Preparation of Quil-A and AVA as a micellar package:

The method used for micelle formation of Quil-A and anti-*V. anguillarum* antibodies was adapted from Jenkins *et al.* (1991). Briefly; micelles were prepared by prolonged vortexing of 10 mg ml^{-1} of purified sheep AVA with 0.03% solution of Quil-A and 0.5 ml of this solution was subsequently administered to fish by mouth.

Preparation of conjugated TraT and LTB with sheep AVA:

The method used for conjugation of anti-*V. anguilarum* antibodies and both TraT and LTB was adapted from Kitagawa *et al.* (1982) as recommended by Biotech Australia with slight modification. A three-step synthesis was used as follows:

1- 1.4 ml of TraT solution (3.6 mg ml^{-1}) dissolved in 4 ml of PBS were incubated with 3.14 mg N-(m-maleimidobenzoyloxy) succinimide (MBS; Pierce, USA) as a

cross-linker dissolved in 0.5 ml of tetrahydrofuran at 30°C for 30 min with occasional stirring. Tetrahydrofuran was removed by flushing with nitrogen, then excess MBS was extracted from the reaction mixture three times with 5 ml portions of methylene chloride. The aqueous layer was used for step 3.

2- Sodium borohydride reduction of disulfide bonds in AVA antibodies:

A total of 25 mg of $NaBH_4$ and 2 ml of n-butanol were alternatively added portionwise to the solution of AVA (10 mg ml^{-1} protein). The mixture was incubated at 30°C for 30 min and then excess $NaBH_4$ was decomposed by adding 5 ml of 0.1 M sodium phosphate (monobasic) and 2 ml of acetone.

3-Conjugation of MBS to the reduced AVA:

The solution of reduced AVA solution was incubated at 25°C for 2 h with the aqueous layer of step 1 solution and the mixture was chromatographed on a Sephadex G-100 column (2.5x57 cm) with 6 M urea as an eluent and concentrated against Aquacide II to yield desirable volume. A 0.5 ml of the chromatographed solution was subsequently administered to each fish by stomach tube.

The same procedure as described above was used for conjugation of sheep AVA to LTB using 10 mg LTB.

ELISA:

An ELISA was set up to determine the activity of immunoglobulins after conjugation in another conjugation trial before the experiment. The reaction was the same as the sheep AVA before conjugation.

Agglutination, precipitation and CFT:

Also agglutination test, precipitation test, complement fixation test (as described in section 3.7.2) were undertaken on the conjugated sheep AVA to assess the activity of Ig. All test proved that conjugation procedure did not affect sheep Ig serological activities.

Trial 1:

Fish:

Rainbow trout average weight 135±14.5 g were used for this experiment. For dosing fish of this size a 1 ml hypodermic syringe was found to be appropriate and used as stomach tube for placing the inoculum into the stomachs of the fish. Some extra rainbow trout were dosed with malachite green and watched for several hours to ascertain there was no regurgitation. All fish were starved for 24 hours before oral immunisation. Fish were all tagged individually in two replicates into two 4000 L temperature controlled (15°C) tanks. Fish were immunised either i.p. with 0.1 ml 100

g^{-1} fish body weight or orally (by stomach tube) with 0.5 ml 100 g^{-1} fish body weight of sheep AVA (Table 3.9.1).

Table 3.9.1: Experimental design for oral immunisation using different carriers

Treatment	Route of administration of antisera	Number of fish(in two replicates) Blood sample* Challenge@ 24 h*	1w*	15 d@	1m*@	2m*	3m*
Purified(Prot.G) sheep AVA	i.p.	12	12	20	32	12	12
Purified(Prot.G) sheep AVA	Stomach tube	12	12	20	32	12	12
Purified(Prot.G) sheep AVA +Quil-A	Stomach tube	12	12	20	32	12	12
Purified(Prot.G) sheep AVA +TraT	Stomach tube	12	12	20	32	12	12
Purified(Prot.G) sheep AVA +LTB	Stomach tube	12	12	20	32	12	12
Control (Untreated tagged)	-	20	-	20	20	-	-
					Total No. fish= 560		

h= Hours post-immunisation Prot.G= Affinity chromatography
w= Weeks " " by Protein G
AVA= Anti *Vibrio anguillarum* Antisera * Fish allocated for blood sampling.
@ Fish allocated for challenge. *@ 12 fish for blood sampling and 20 fish for challenge

At each interval the required number of immunised fish (from those fish dedicated for serological tests) were taken out for blood sampling. Blood samples were collected via the caudal vessels and fish were euthanised after each blood collection.

Fish sera collected at 24 h, 1 m, 2 m and 3 m (Table 3.9.1) were examined by a specific ELISA using carriers Quil-A, TraT and LTB as antigen for coating ELISA plates for any response against Quil-A, LTB and TraT. Since they are immunostimulants it was postulated that fish could produce antibody against them.

Also ELISAs were run to determine if fish responded to sheep Ig conjugated to the three carriers, using sheep Ig as antigen for coating ELISA plates. Since carriers used for transporting these antibodies were reported to have all have immunostimulatory properties and it was presumed that sheep AVA to have been transferred into fish through the intestine. So it was possible that fish responded significantly to sheep AVA.

Trial 2:

In order to evaluate the immunostimulatory effect of Quil-A, TraT and LTB themselves on fish, groups of fish were dosed by these carriers with the same amount used for conjugation with sheep AVA (Table 3.9.2).

Table 3.9.2: Experimental design for oral dosing using different carriers only

Treatment	Route of administration	Number of fish challenged(in two replicates)	
		Replicate 1	Replicate 2
Saline	Stomach tube	10	10
Quil-A 0.03% solution	Stomach tube	10	10
TraT 50 µg/fish	Stomach tube	10	10
LTB 100 µg/fish	Stomach tube	10	10

Virulent *V. anguillarum* with LD_{70} for challenge:

Fish were challenged with the virulent bacteria as described in section 2.10.1. In this experimental challenge, fish were inoculated with 7.1×10^7 cfu 0.1 ml^{-1} (LD_{80}), 7×10^7 cfu 0.1 ml^{-1} (LD_{75}) and 7.4×10^7 cfu 0.1 ml^{-1} (LD_{85}) of live *V. anguillarum* cells at 15 days, 30 days and 15 days (carriers only) challenges respectively.

3.10.3 **Results:**

Trial 1:

Fifteen-day challenge:

With respect to the protection conferred by oral immunisation using three carriers, the highest protection was observed in fish fed AVA conjugated to LTB by stomach tube (RPS= 37.5%). Results of challenge after 15 days of immunisation for determination of RPS is shown in table 3.9.3.

Table 3.9.3: Mortalities and RPS after challenge (with virulent *V. anguillarum*, LD_{80}) of rainbow trout passively immunised [(orally and by injection using Protein G purified sheep AVA (SAVA)] at 15 days p.i. as compared to controls

Treatment	Mortalities caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
SAVA(0.1 ml) by injection	0/10	0/10	0/20	100 ± 0.0
SAVA(0.5 ml) by stomach tube	7/10	9/10	16/20	0.0 ± 6.25
SAVA+Quil-A (0.5 ml) by stomach tube	8/10	7/10	15/20	6.2 ± 6.25
SAVA+TraT (0.5 ml) by stomach tube	6/10	7/10	13/20	18.7 ± 6.25
SAVA+LTB (0.5 ml) by stomach tube	5/10	5/10	10/20	37.5 ± 0.0
Control Non-injected tagged fish	8/10	8/10	16/20	0.0 ± 0.0

* Experimental challenge $LD_{80}=7.1x10^7$ cfu 0.1^{-1} ml by i.p. injection.

Groups of fish fed AVA conjugated to TraT or in micellar form with Quil-A showed RPS of 18.7% and 6.2% respectively. However, protections obtained by oral immunisation were not significantly different from control ($P< 0.05$). This is while the protection in fish injected with the purified sheep AVA had a RPS of 100%. No protection was conferred following oral dosing of fish with sheep Ig.

Thirty-day challenge:

Results of challenge immunised fish after 30 days by oral route is shown in table 3.9.4.

Table 3.9.4: Mortalities and RPS after challenge (with virulent *V. anguillarum*, LD_{75}) of rainbow trout passively immunised [(orally and by injection using Protein G purified sheep AVA(SAVA)] at 30 days p.i. as compared to controls

Treatment	Mortalities caused by *V. anguillarum**			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
SAVA (0.1 ml) by injection	1/10	0/10	1/20	93.3 ±6.67
SAVA (0.5 ml) by stomach tube	8/10	8/10	16/20	0.0 ± 0.0
SAVA+ Quil-A (0.5 ml) by stomach tube	8/10	9/10	17/20	0.0 ± 0.0
SAVA+ TraT (0.5 ml) by stomach tube	7/10	6/10	13/20	13.3 ± 6.67
SAVA+ LTB (0.5 ml) by stomach tube	6/10	5/10	11/20	27.0 ± 7.0
Control Non-injected tagged fish	7/10	8/10	15/20	0.0 ± 6.67

* Experimental challenge $LD_{75}=7x10^7$ cfu/ 0.1 ml i.p. injection

The highest protection after 30 days post-immunisation was observed in fish fed AVA conjugated to LTB by stomach tube (RPS = 27%). This was not significantly different from the RPSs of AVA conjugated Quil-A, TraT and control fish ($P< 0.05$). Groups of fish fed AVA conjugated to TraT or in micellar form with Quil-A showed RPS of 13.33% and no relative protection respectively.

Trial 2:

Challenge of fish 15 days after dosing carriers only:

Results of challenge on fish dosed with only carriers without immune sera is shown in table 3.9.5.

Table 3.9.5: Mortalities and RPS after challenge (with virulent *V. anguillarum*, LD_{80}) of orally dosed rainbow trout using different carriers only at 15 days p.i. as compared to controls

Treatment	Mortalities caused by *V. anguillarum**			RPS
	Replicate 1	Replicate 2	Combined Replicates	
Quil-A0.03% solution by stomach tube	9/10	9/10	18/20	0
TraT (TraT) 50µg/fish (0.5 ml) by stomach tube	8/10	9/10	17/20	0
LTB 100µg/fish by stomach tube	9/10	9/10	18/20	0
Control Non-injected tagged fish	8/10	9/10	17/20	0
Saline by injection	8/10	9/10	17/20	0

* Experimental challenge $LD_{85}=7.4x10^7$ cfu 0.1 ml $^{-1}$ by i.p. injection

No protection was observed following the oral dosing of the different carriers (Quil-A, TraT and LTB) alone.

ELISA:

The ELISA for monitoring the AVA levels transferred orally into the fish after different intervals (24 h, 1 week, 15 days, 1 month, two months) post-immunisation were all negative (O.Ds ranged from 0.092- 0.135). Nonetheless, serum samples of fish injected intraperitoneally with sheep AVA showed significant antibody titre at 24 h after immunisation and to a lesser extent later. The rate of clearance of purified AVA injected to the fish was similar to the rate of clearance of the whole AVA (without any purification) that was measured earlier (section 3.2.3).

The ELISA for monitoring fish anti-sheep Ig antibodies against protein-G purified antibodies was carried out, however, all ELISA results showed negative values (O.Ds in a range of 0.085- 0.128) indicated low uptake by fish and/or low immunogenicity for fish. Figure 3.9 shows the relative survival of fish orally dosed with AVA conjugated to carriers after 15 and 30 days post-immunisation.

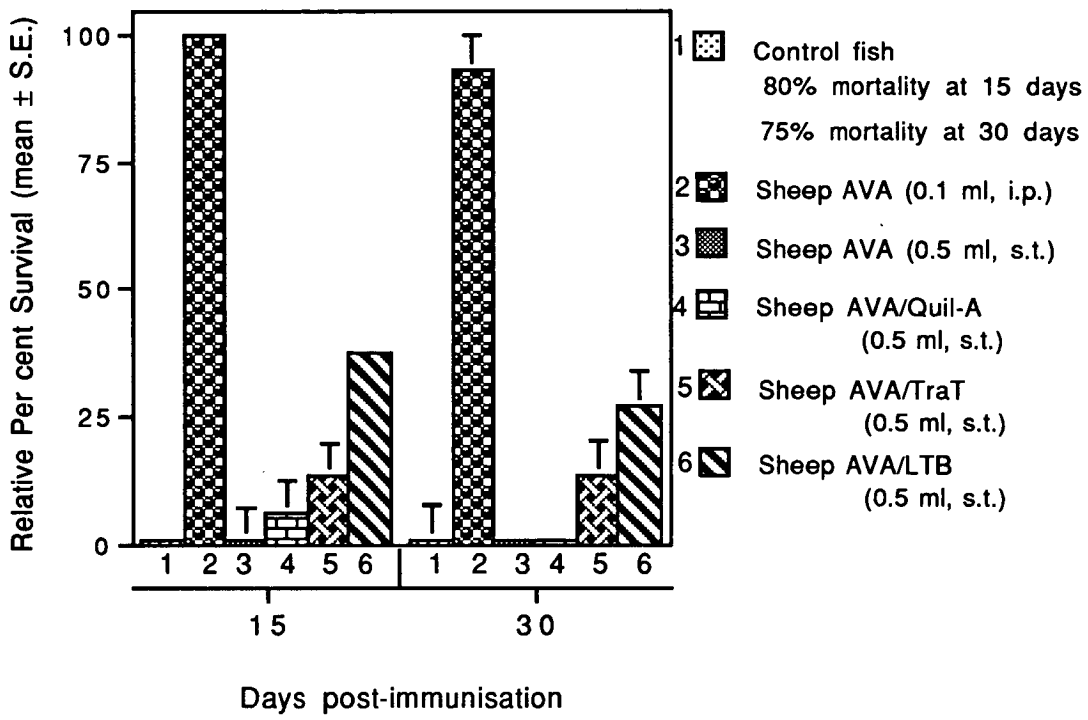


Figure 3.9: Protection conferred by conjugated sheep AVA dosed orally to fish

3.10.4 **Discussion:**

In groups of fish orally immunised by AVA conjugated to different carriers, the highest protection was seen in fish fed AVA conjugated to LTB by stomach tube (RPS= 37.5% at 15 days after dosing) suggesting a moderate to marginal protection following conjugating AVA with this carrier. Groups of fish fed AVA conjugated to TraT and micellar form of Quil-A only showed RPS of 18.7 and 6.25% respectively.

The highest protection at 30 days post-immunisation was observed in fish fed AVA conjugated to LTB by stomach tube (RPS = 27%) that marginally protected fish. These results demonstrate that oral vaccination may be a feasible approach for the prevention of fish diseases. This presumably suggests that the specific antibodies transferred to fish bound to receptors at key sites such as the spleen, liver and kidney. Groups of fish fed AVA conjugated to TraT or in micellar form with Quil-A showed RPS of 13.33% and no protection respectively (Figure 3.9). A possible explanation for low level or no protection obtained in this experiment might be the method of conjugation. Further research is required to elucidate this.

Feeding of different carriers themselves did not confer any protection to the fish as a result of their immunostimulatory activities. A possible explanation is that the carriers had a low uptake in the dosage used for fish in this experiment.

In group of fish immunised passively by intraperitoneal injection, complete protection (RPS = 100%) was found in the group of fish immunised with purified

sheep AVA 15 days and significant protection (RPS = 93.33%) one month post-immunisation. This is consistent with the results of Maquis and Lallier (1989) who found that purified rabbit immunoglobulins specific to virulent *A. salmonicida* were as protective as the initial rabbit hyperimmune serum in protecting brook trout against furunculosis.

In challenge of immunised fish, a single i.p. dose of *V. anguilarum* was used. It was shown earlier that the virulent bacteria appears in the circulating water soon after the challenge and a permanent exposure of fish to the organism continues. The rate of mortality of control fish cohabited (horizontal infection) with immunised challenged fish due to this exposure was 20%. Therefore, fish must have a certain degree of immunity to resist the infection regardless of the route of exposure. For example immunised fish with a certain specific antibody level, memory cells or non-specific immunity can only survive.

The rate of elimination of purified sheep AVA injected to the fish was similar to the rate of decay of the whole AVA (without purification). This explains the longevity of protection provided by the specific immunoglobulins in both whole and purified sera.

No protection was observed following the feeding of different carriers(Quil-A, TraT and LTB) by stomach tube. These show that protection obtained by oral immunisation of AVA conjugated to these carriers was due to specific anti-*Vibrio anguillarum* antibodies conferred to the fish. Secondly, these results indicate that the protection provided by these carriers was not due to their immunostimulants activity, at least when they are used against vibriosis.

Radiolabelled AVA might be considered as a sensitive indicator of AVA in fish serum and tissues in order to elucidate the sites of activity of orally-administered AVA. Also, dosing fish with higher amount of carriers may enhance greater gastrointestinal absorption of TraT and LTB.

CHAPTER 4.0

STREPTOCOCCOSIS, PASSIVE AND ACTIVE IMMUNISATION AND EVALUATION OF PROTECTION

4.1 Introduction/Literature review:

Streptococcosis has been reported in a number of species, but the specific disease has only been definitely identified in Japan among yellowtails (*Seriola quinqueradiata*) (Kusuda *et al.*, 1976; Kitao *et al.*, 1979), in Japanese eels (Kusuda *et al.*, 1978b), ayu and tilapia (Kitao *et al*, 1981); the populations of rainbow trout in South Africa (Boomker *et al.*, 1979), Australia (Carson and Munday, 1990) and Italy (Ceschia *et al.*, 1992). The disease may also have occurred sporadically in Great Britain and Norway (Austin and Austin, 1993). Nevertheless, there is good evidence that streptococcosis is problematical in both farmed and wild fish stocks (Austin and Austin, 1993).

While the taxonomy of the organism has not been defined, it is presently classified as an *Enterococcus* sp., probably *Enterococcus seriolicida* (Kusuda *et al.*, 1991).

Streptococci are gram-positive and spherical (approximately 0.5 to 1.0 μ in diameter), usually non-motile and non-spore forming bacteria. They usually grow in pairs or characteristically form chains. Most of them grow on common laboratory media at temperatures between 20° and 37°C, but addition of blood or tissue extracts enhances their growth.

Many of the pathogenic streptococci may be divided on the basis of serological differences, based on the composition of antigens located in their cell walls and a combination of features: colony growth characteristics, haemolysis patterns on blood agar (α-haemolysis, ß-haemolysis) and biochemical reactions. For example, the ability to attack blood has been highlighted since pathogenic strains show different patterns on blood agar; i.e, α-haemolytic (Kusuda *et al.*, 1976), ß-haemolytic (Robinson and Meyer, 1966; Minami *et al.*, 1979; Kitao *et* al., 1981), non-haemolytic (Plumb *et al.*, 1974; Cook and Lofton, 1975; Iida *et al.*, 1986). Although, some well-established taxa, e.g. *Streptococcus agalactiae* contain both α and ß-haemolytic strains (Cowan, 1974).

At various times, the fish pathogenic streptococci have been linked with *Streptococcus agalactiae, S. dysagalactiae, S. equi, S. equisimilis, S. faecium, S. pyogenes* and *S. zooepidermicus*. South African isolates have been described as comprising unidentified Lancefield Group D *Streptococcus* (Boomker et al., 1979); Japanese isolates have been linked with, but are not identical to *S. faecalis* and *S. faecium;* whereas American strains approximate to the description of *S. agalactiae* (Kusuda and Komatsu, 1978).

Kitao *et al.* (1981) reported a new serotype in Japan which did not react with specific antisera to Lancefield groups. This conclusion was also reached by Kitao (1982b) and Kusuda and Kimura (1982).

A significant number of works describe the isolation and some characteristics of streptococci isolated from diseased fishes. Many characteristics are shared by the majority of fish pathogenic streptococci. However, there is some variance in description reported by workers. For example, biochemical reactions and growth temperatures of South African and Japanese strains. Such differences may reflect the lack of standardisation in the tests and heterogenecity in the species composition of the pathogens isolated from the fish (Austin and Austin, 1993).

Properties of 12 strains of pathogenic streptococci from yellowtails and eels in Japan and 4 strains from saltwater fishes and white bass in America were compared by Kusuda and Komatsu (1978). Japanese isolates have been linked with, but are not identical to *S. faecalis* and *S. faecium*; whereas American strains approximate to the description of *S. agalactiae*.

Kusuda *et al.* (1991) described *E. seriolicida* sp. *nov.*, isolated from diseased specimens of cultured yellowtail and eels as a result of detailed biochemical and DNA/DNA hybridisation studies. The bacterium was gram-positive, short-chain-forming, catalase-negative, a facultatively anaerobic coccus, grew at 10 and 45°C in 6.5% NaCl (pH 9.6) with 40% bile and 0.1% methylene blue-milk were positive. It could be distinguished from other species of the genus *Enterococcus* by several biochemical characteristics and by Lancefield's group antigen. The researchers indicated that the isolates belonged to the genus *Enterococcus* and that they could be differentiated from *Streptococcus* sp. and *Lactococcus* spp. by positive growth at both 10 and 45°C, in 6.5% NaCl, and at pH 9.6, which are important characteristics of *Enterococcus* sp. (Schleifer and Kilpper-Balz, 1987). Therefore, "enterococcal infection" is proposed instead of "streptococcal infection" or "streptococcosis" for the disease caused by this ethiological agent (Kusuda *et al.*, 1991).

4.1.1 **The streptococcal fish pathogen used in this study**:

In a recent study, Australian and South African strains collected from outbreak of streptococcal disease from farmed rainbow trout, appeared to have attributes of the genus *Enterococcus*. Although no Lancefield group D antigen could be detected, all strains grew at 10°C but not at 45°C, grew in 6.5 % NaCl, were bile-aesculin positive and hydrolysed L-pyrrolidonyl-ß-naphthylamide and had similar phenetic characteristics and whole cell protein profiles (Carson *et al.*, 1993). *Streptococcus* sp. biovar. I, has been reported the etiological agent of streptococcosis in Australia (Carson, 1990). All workers recommended more work to resolve the exact status of fish pathogenic enterococci/ streptococci. The epithet *Streptococcus* sp. (biovar 1) is used in the present study. This isolate is α-haemolytic on sheep RBC, Voges-Proskauer reaction positive, hydrolyses aesculin and grows in 0-6.5% NaCl.

4.1.2 **Epidemiology**:

Streptococcal infection has been described as a serious disease of cultured fish. The disease transmitted horizontally within 5 days by cohabiting an infected golden shiner with healthy specimens of the same species (Robinson and Meyer, 1966). Food-borne infection was highlighted by Taniguchi (1983).

Streptococcal infection may cause serious problems in both fresh water and marine environments. For instance, in Japan, streptococcal infections occur among many different species of fish especially yellowtail (*Seriola quinqueradiata*) (Kusuda et al., 1976; Minami *et al.*, 1979) and eels (*Anguilla japonicus*) (Kusuda *et al.*, 1978b). It has been shown that the bacteria remain in sea water and mud around fish farms throughout the year, but during the summer months somewhat higher numbers of organisms are detected in sea water than in mud (Inglis *et al.*, 1993). In contrast, during autumn and winter, Kitao *et al.* (1979) found that the organisms were more easily isolated from mud.

Streptococcosis has been associated with acute to chronic mortalities of several estuarine fishes (striped mullet, Atlantic menhaden, pinfish, Atlantic croaker, sea trout) in coastal Alabama and Florida (Plumb *et al.*, 1974).

In fresh water it has been reported in rainbow trout (Hoshina *et al* , 1958; Barham *et al* ., 1979; Miyazaki, 1982). Kitao *et al.* (1981) isolated streptococci from several species of tilapia. The *Streptococcus* sp. isolated from fish in Japan was haemolytic.

In North America, non-haemolytic Group B streptococci were isolated from diseased golden shiners, (*Notemignus crysoleucas*) (Robinson and Meyer, 1966) in freshwater and several species of marine fishes from the gulf of Mexico (Plumb *et al.*, 1974). These two isolates were serologically identical.

Carson and Munday (1990) described streptococcosis as an emerging disease in aquaculture and pointed out the wide geographic distribution around the world. They also emphasised the fact that many varieties of fish are at the risk of the disease. In water temperature in excess of 18°C, more mortality was observed (up to 60%).

4.1.3 **Pathogenicity mechanisms**:

Experimental infections have been achieved by injection of 10^4 to 10^5 cells (Cook and Lofton, 1975), and by exposure (immersion) of fish for 10 min to 10^6 bacteria (Robinson and Meyer, 1966). The pathogenicity of α-haemolytic *Streptococcus* species towards yellowtail is enhanced by *in vivo* passage through yellowtail (Kusuda and Kimura, 1978). After percutaneous challenge with bacteria of high virulence, the kidneys retained the bacteria with the relatively high count of 10^7 cells per gram of tissue while in other organs 10^5-10^6 cells were detected in 10 min, this was followed by a progressive decrease up to 24 h post-inoculation with a subsequent rapid increase during the later stages of the disease progress. The highest rate of growth was obtained in the intestine, where 10^7 cells were detected at 72 h

after inoculation. After oral challenge, the bacteria were detected at high levels from organs and blood within 10 min but they were completely removed from all organs except the intestine within 24 h (Kusuda and Kimura, 1978).

Kimura and Kusuda (1979) isolated *Streptococcus* sp. from blood and various internal organs of cultured yellowtails shortly after challenge irrespective of the virulence of bacteria or route of infection. The pathogenicity of streptococci in yellowtail has been attributed to an exotoxin (Watson, 1960).

The toxicity of the ECP and it's relation to the virulence factors of *Streptococcus* sp. was tested (Sakai *et al.*, 1991b). However, they could not determine the main virulence factor. Only 20% of rainbow trout injected with 10-30 mg of ECP died. Nevertheless, in all injected fish, haemorrhages and inflammation of the injected sites were observed.

Pathogenicity of a non-haemolytic group B *Streptococcus* sp. in gulf killifish (*Fundulus grandis*) has been studied by Rasheed and Plumb (1984). Fish were infected when they were injured prior to dipping in to the bacterial suspension. Oral administration of the bacteria, crowding of fish and low oxygen concentration after dipping into the bacterial suspension did not precipitate infection. No death was reported by dipping alone in the bacterial suspension nor when the fish were exposed to a 50 parts per thousand hyperosmotic solution prior to dipping.

Regarding antigenic variation, Kitao (1982b) stated that there were two antigenic types; the KG+ type agglutinates and KG- type strain possessed a specific envelope-like substance which inhibits agglutination with antiserum of the *Streptococcus* KG7409.

Streptococcosis has been reported in a number of fish species including bait minnow (golden shiners) in the United States, rainbow trout in Japan, South Africa and Australia, yellowtail, red sea bream, ayu, eel, and true sardine (*Sardinopus melanosticta*) (Japan), while some tilapia species (*Sarotherodon mossambicus* and *Tilapia sparrmanii*), carp and largemouth bass (*Micropterus salmoides*) were not affected (Boomker *et al.*, 1979). Comparative studies indicated that, rainbow trout were much more susceptible to streptococcosis than either Atlantic salmon or brown trout (Munday *et al.*, 1993). Mortality in rainbow trout was closely related to water temperatures in excess of 18°C (Munday *et al.*, 1993).

4.1.4 **Clinical signs and pathology**:

Fish with streptococcal septicemia may become lethargic. Erythema around the anus, inflammation along the dorsal surfaces especially in the vicinity of the caudal peduncle are frequently observed. Haemorrhages in the intestine and the existence of pink mucus which fills the intestine, swollen kidney , congested liver with dark colour and inflammation in the muscular tissues are other gross lesions due to the disease.

Streptococcal infection produces a typical septicemia, pronounced exophthalmos (popeye) often leading to complete degeneration of the eye and

asymptomatic carriers. In acute cases fish become dark, swim sluggishly and cease feeding. Chronically infected fish may develop a scoliosis (twisting of the spine) (Jack, 1990). No *Streptococcus* sp. has been isolated from either culture of the egg surface or the interior of the egg. Thus it appears that the disease is not vertically transmitted (Jack, 1990).

Variation in disease symptoms include exophthalmos and distended abdomen (also common features of bacterial kidney disease), haemorrhaging in the eye (enteric red mouth feature), haemorrhages in the opercula, at the base of the fins, and haemorrhages on the surface (similar to vibriosis). Darkening of the skin and erratic swimming of the fish are other characteristics of the disease.

Postmortem findings in yellowtail were damage to the liver, kidney, spleen and intestine. Also, abdominal cavity filled with ascitic fluid, enteritis, pale livers and blood in the peritoneal cavity were seen. Kidneys were unaffected (Plumb *et al.*, 1974).

4.1.5 **Isolation of the pathogen**:

Recovery of the streptococcosis is straightforward, involving use of bovine blood tryptose agar (Boomker *et al.*, 1979), Todd-Hewitt broth, nutrient agar supplemented with rabbit blood (Kitao *et al.*, 1981) or brain heart infusion agar (Minami *et al.*, 1979).

Inoculated media (incubated at 22-37°C for up to 48 h) develop dull grey colonies approximately 1-2 mm in diameter. Confirmation of the presumptive identification with different biochemical and serological tests is necessary

The growth of this organism at an incubation temperature of 37°C is in excess of the preferred temperatures of many fish species, particularly salmonids. This may indicate that *Streptococcus* sp. have been derived from warm-blooded animals, and may therefore constitute a public health risk. This aspect is presently under investigation (Hawkesford, pers. comm.).

Isolation and identification of the bacterium is necessary for definitive diagnosis. The use of special media with different amount of sodium chloride, bile salts and other substances such as various carbohydrates leads to the definite recognition of the organism. Bragg *et al.* (1989) described a selective media for the field isolation of pathogenic *Streptococcus* sp. of rainbow trout.

4.1.6 **Treatment**:

Apart from good management, including the prompt removal and destruction of diseased fish and the adoption of improved hygienic practices around fish farms, use of antimicrobial compounds have met with considerable success. Effective antimicrobial compounds for the treatment of streptococcosis in fish are listed in Table 4.1.

Table 4.1: Antimicrobial compounds for streptococcosis treatment

Antimicrobial compound	Dose	Reference
Erythromycin	25 mg/kg body weight of fish/day for 4-7 days	(Kitao, 1982a)
	75-125mg/kg body weight /day for 10-21 days	(Munday, 1993)
Doxycycline	20 mg/kg body weight of fish/day	(Nakamura, 1982)
Josamycin	30 mg/kg body weight of fish for 3 days (or at 20 mg/kg body weight of fish/day for 5 days)	(Kusuda and Takemaru, 1987; Takemaru and Kusuda 1988)
Sodium nifurstyrenate	50mg/kg body weight of fish per day for 3 to 5 days	(Austin and Austin, 1993)
Oxytetracycline	50-75 mg/kg of body weight/day for 10 days	"
Tetracycline	75-100 mg/kg of body weight/day for 10-14 days	"

4.1.7 **Disease control:**

The development of vaccine against streptococcosis of cultured yellowtail has been investigated. Kusuda and Takagi (1983) determined antibody production against *Streptococcus* sp. in naturally infected yellowtail by agglutination and passive hemagglutination of endotoxin and exotoxin of the bacteria. It was implied in this work that the toxoid vaccine was better than the bacterin because the antibody production against endotoxin was greater than that which was produced against the cellular antigen in yellowtail. Therefore, it was necessary to examine the toxicity and detoxication of the endotoxin to yellowtail in order to develop the vaccine for streptococcosis. This is despite the fact that it is generally believed that gram positive bacteria do not possess endotoxin. Production of agglutinating antibody in intestinal mucus suggests that the intestine is an important place for fish to develop this infection first (Kusuda and Takagi, 1983). However, attempts to introduce a commercial vaccine against the disease have not yet been successful (Carson and Munday, 1990).

4.1.7.1 **Immunisation:**

Vaccination as a control measure has been recommended (Iida *et al.*, 1981). Sakai *et al.* (1987) vaccinated rainbow trout against ß-haemolytic streptococcal disease. They used formalin killed cells with and without Freund's complete adjuvant, both intraperitoneally and by immersion. Their results showed that following challenge with live bacteria 70% or greater protection was achieved. Nevertheless, anti-*Streptococcus* sp. agglutinating antibody titres were low in intraperitoneally immunised fish and no detectable agglutinating antibody was demonstrated following the immersion route.

The opsonic effect of antibody and complement on phagocytosis of *Streptococcus* sp. by macrophage-like cells of yellowtail has been investigated. Specific antibody increased the phagocytic activity and phagocytic index significantly, However, after addition of complement neither phagocytic activity nor the phagocytic index changed significantly. In the presence of specific antibody and complement, phagocytic activity and phagocytic index were significantly higher than in the controls. It was concluded that activation of the classical pathway of yellowtail complement by antigen-antibody reaction is an important function in opsonisation of particulate antigen (Kusuda and Tanaka, 1988).

4.2 Experiment I: Passive immunisation (streptococcosis):

(determination of the rate of clearance of ASA from fish sera)

4.2.1 Introduction and Aims:

Passive immunisation of fish against furunculosis, vibriosis, edwardsiellosis etc. have been tried to some extent (Table 1.1.1 section 1.1). However, a study to research aspects of passive immunisation against streptococcosis particularly collecting immunised fish sera at different intervals and monitoring the rate of clearance of them from fish sera by ELISAs has not been reported in the literature. The effectiveness of active immunisation against streptococcosis has yet to be established. Therefore, passive immunisation might be an alternative method.

The aim of this study was to measure the rate of elimination of transferred anti-*Streptococcus* sp. antibodies from fish sera. In this experiment fish in two replicates were injected intraperitoneally with three types of immune antisera (sheep, rabbit and fish anti-*Streptococcus* antibodies).

4.2.2 Materials and Methods:

Antisera:

Sheep, rabbit and fish anti-*Streptococcus* sp. (ASA) were prepared as described in section 2.3. The ELISA antibody levels of sheep and rabbit ASA were 2.10 and 0.637 respectively at dilution of 1:128 and that of fish (ASA) was 0.301 at dilution of 1: 64. Fish were immunised with 0.1 ml 100 g^{-1} of their body weight.

Fish:

Rainbow trout (mean weight 57± 6.6 g) were tagged and treated according to the experimental design (Table 4.2.1). Eighty fish were allocated randomly for sheep, rabbit and fish ASA injection groups. Fish were lightly anaesthetised and immunised at the start and kept in two large fish bins (400 L with a constant temperature of 15°C) (each fish bin for one replicate). After 2, 4, 6 and 24 h intervals, 20 fish were bled and returned to main tanks in such a way that at 24 h after immunisation all the fish had been bled and placed in two temperature-controlled tanks (15°C). After 1 week, 1, 2, 3 m, each time 24 fish of each group were taken from the main tanks, bled and returned to the tank. In addition 20 fish were bled from stock at the start of the experiment and their sera were used as negative control in ELISA.

ELISA

ELISA was run according to the ELISA protocol established and described in section 2.6.4. Sera collected from passively immunised fish at different intervals were tested by the specific ELISA against sheep and rabbit antisera already described in section 2.6.3.1 in order to measure the heterogenicity of these antisera for fish.

Table 4.2.1: Experimental design for monitoring the rate of clearance of anti-*Streptococcus* sp. antibodies (ASA) from fish sera

Treatment	Route of administration	Number of fish (in two replicates) bled at							
		2,	4,	6,	24 h	1w	1m	2m	3m
Sheep ASA	i.p.	20	20	20	20	24	24	24	24
Rabbit ASA	i.p.	20	20	20	20	24	24	24	24
Fish ASA	i.p.	20	20	20	20	24	24	24	24
Control	-	20							
						Total No. fish= 260			

h= Hours post-immunisation w= Weeks post-immunisation m= month

4.2.3 Results:

Administration of sheep anti-*Streptococcus* sp. antibodies (ASA) was followed by a significant elevation of the immune antibodies in fish sera at 2, 4, 6 h post-immunisation. The peak ELISA value (O.D= 0.921) for these antibodies was 24 h after immunisation. Sheep antibody levels declined slightly at one week (mean O.D= 0.901) and more after one month (mean O.D= 0.392) post- immunisation. Sheep ASA showed an elevated ELISA activity (O.D= 0.244) after 2 months and then appeared to be nearing a low level (O.D= 0.187) at three months post-immunisation which was not significantly different from that of control fish ($P< 0.05$) (Figure 4.2.1).

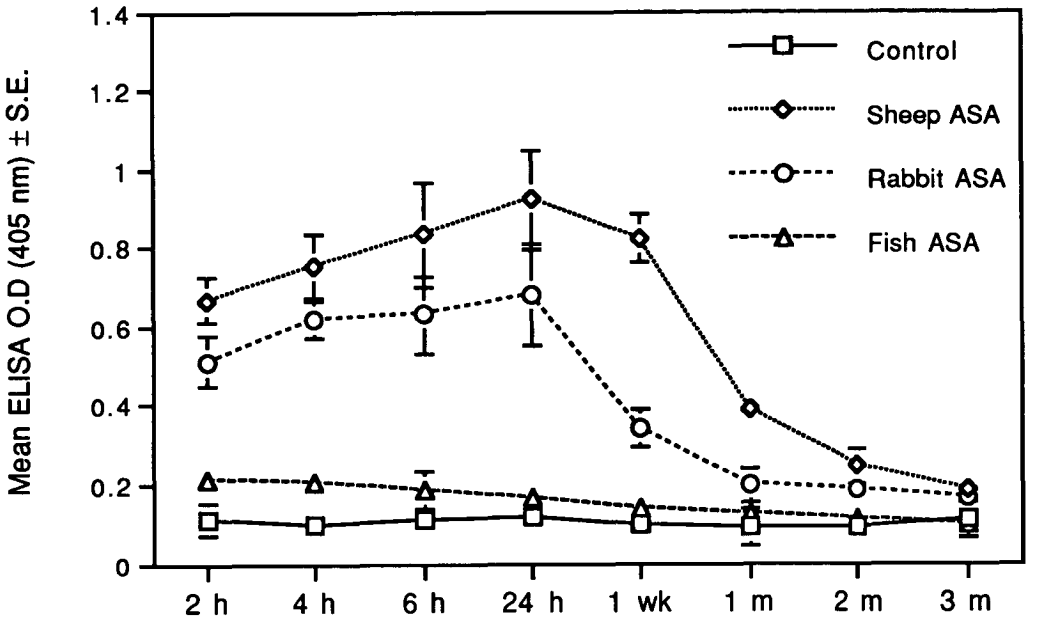


Figure 4.2.1: Rate of clearance of three types of anti-*Streptococcus* sp. antibodies from fish sera

Fish given rabbit anti-*Streptococcus* sp. antibodies showed a significant elevation in serum antibody level measured by ELISA 2 h post- immunisation (O.D= 0.511). The antibody level continued to rise up to 24 h (mean ELISA O.D= 0.680) after immunisation and declined gradually by week 1 (mean ELISA O.D= 0.341). At one month post-immunisation, the antibodies level had declined markedly and the mean ELISA O.D was 0.199 (significant differences are shown in Table 4.2.2). From this point onward the ELISA antibody levels were similar to those of control fish (Figure 4.2.1).

Fish passively immunised by fish ASA antibodies showed the highest amount of antibodies at 2 h post-immunisation (O.D= 0.221). These antibodies declined rapidly up to 24 h when no positive O.D could be detected. ELISA O.Ds were the same as control samples from 24 h onwards. Summary of the Figure 4.2.1 and the significant values are shown in Table 4.2.2.

Table 4.2.2: The summary of figure 4.2.1; rate of clearance of ASA from fish serum

Time post-passive immunisation	24h	1 week	1 month	2 months	3 months
	ELISA O.D readings (mean ± S.E.)*				
Control	$0.121^{a}\pm0.025$	$0.119^{a}\pm0.034$	$0.091^{a}\pm0.046$	$0.094^{a}\pm0.023$	$0.109^{a}\pm0.042$
Sheep ASA	$0.921^{b}\pm0.125$	$0.901^{b}\pm0.063$	$0.392^{c}\pm0.031$	$0.244^{c}\pm0.44$	$0.187^{ac}\pm0.032$
Rabbit ASA	$0.680^{d}\pm0.126$	$0.341^{c}\pm0.048$	$0.199^{ac}\pm0.044$	$0.184^{ac}\pm0.035$	$0.163^{a}\pm.032$
Fish ASA	$0.165^{a}\pm0.027$	$0.141^{a}\pm0.027$	$0.124^{a}\pm0.032$	$0.111^{a}\pm0.020$	$0.101^{a}\pm0.024$

*Values with the same superscript were not significantly different ($P<0.05$).

The highest ratio of antibody level of immunised fish with sheep ASA to non-immunised fish was 7.6 times at 24 h and 7.5 times at 1 week post-immunisation. This ratio was 5.6 and 1.6 times after 24 h for fish immunised with rabbit ASA and fish ASA respectively (Table 4.2.3).

Table 4.2.3: The ratio of O. D of immunised fish relative to O. D of non-immunised fish (control fish) at each observation

Time post-passive Immunisation	24h	1 Week	1 month	2 months	3 months
Sheep ASA	7.611	7.571	4.296	2.595	1.715
Rabbit ASA	5.619	3.376	2.186	1.968	1.495
Fish ASA	1.633	1.396	1.362	1.191	0.926

Fish antibody responses to sheep and rabbit ASA are shown in Figure 4.2.2. The highest response was 2 months after immunisation of fish with sheep and rabbit sera. There was no significant difference between fish response to rabbit sera and sheep sera at each interval ($P< 0.05$).

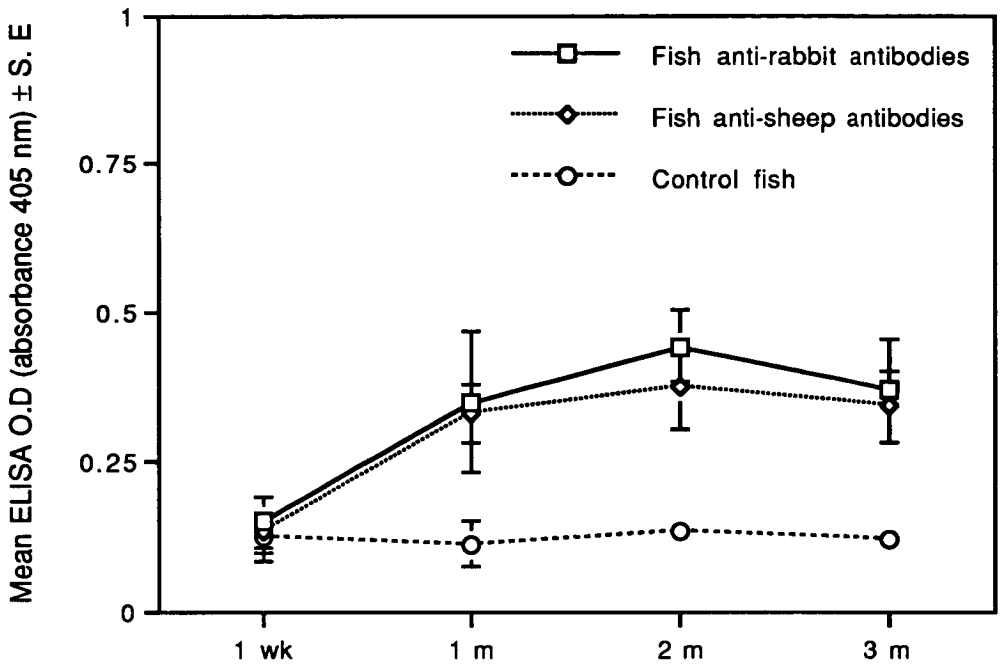


Figure 4.2.2: Fish humoral response to sheep and rabbit anti-*Streptococcus* sp. antibodies

4.2.4 Discussion:

In this experiment, sheep ASA antibodies reached the highest amount in fish sera after i.p. administration with the peak occurring at 24 h post-immunisation. The antibody peak for rabbit ASA and fish ASA post-immunisation occurred at 24 h and 2 h respectively. The persistence of antibody appeared to be highest after administration of sheep antibodies. There were significant differences in the level of ASA in fish injected with sheep and rabbit ASA at different intervals post immunisation (Table 4.2.1) which shows the persistence of sheep ASA.

The patterns of appearance of sheep and rabbit antibodies from 2 to 24 h could well be explained by the gradual appearance of these antibodies in fish blood. Nonetheless, fish ASA shows the highest amount only two hours post-immunisation. This is probably due to the low amount of these antibodies in fish immune sera. Fish injected with fish ASA showed comparatively lower amounts of these antibodies post-immunisation which was significantly different from groups of fish injected with sheep and rabbit ASA ($P<0.05$). It seems that rabbit ASA was metabolised rapidly whereas sheep ASA persisted much longer. This might be due to the fish responses to the heterogenous rabbit Ig.

It is possible that because sheep and rabbit responded greater than fish to *Streptococcus* sp., their immunised sera could confer a higher level of immunity which

is more prolonged. In general the sheep ASA shows a high persistency followed by rabbit ASA in fish blood.

4.3 Experiment II Active immunisation (streptococcosis):

4.3.1 Introduction and Aims:

Active immunisation of fish was already discussed in section 4.1.9. Since there is no bacterin available for the control of streptococcosis and also the fish responses to bacterins have not been clearly elucidated, the intension was to study the use of bacterin by i.p. and immersion method and demonstrate the fish response to the Australian *Streptococcus* sp. isolated from rainbow trout in Tasmania.

4.3.2 Materials and Methods:

Bacterins:

The method used in preparation of *Streptococcus sp.* bacterins was as described in section 2.2. One group of fish with a replicate was injected i.p. with 0.1 ml of bacterin containing 1 mg washed formalin killed bacteria (dry weight) using a 1 ml hypodermic syringe. The other group of fish with a replicate was immersed for 30 seconds in bacterin as described in section 2.2.2. The immersion vaccination method consisted of placing the netted fish in a aerated 20 L fish box containing 10% diluted bacterin (18 L freshwater + 2L bacterin). Each ml of vaccine solution contained 5.5 x 10^9 formalin killed bacteria. The fish were placed in the vaccine for 30 sec then washed in a fish box containing aerated freshwater for 1 min and then returned to main tanks. Temperature of the vaccine solution was kept at the ambient water temperature.

Fish:

Sixty four rainbow trout (average weight of 57± 6.6 g) were used as shown in the experimental design (Table 4.3.1). Twenty four fish were immunised in each group and they were divided randomly in two replicates into two temperature controlled tanks. Fifteen fish were allocated for a control group to collect serum samples for negative ELISA controls. Fish were bled one month and again 2 months post-immunisation.

Table 4.3.1: Experimental design for active immunisation against *Streptococcus* sp.

Treatment	Route of administration	Total number of fish	Number of fish (in two replicates) Blood sample	
			1m	2m
Immersion vaccinated	immersion (30 s)	24	24	24
Injected vaccinated(1 mg cells)	i.p.	24	24	24
Control	-	16	12	12
			Total No. fish= 65	

ELISA

ELISA was run according to the protocol described in section 2.6.4.

4.3.3 Results:

The results of active immunisation are summarised in Table 4.3.2. Immersion vaccinated fish did not show any antibody level 1 or two months post-immunisation. Fish injected with 1 mg formalin killed *Streptococcus* sp. cells showed an antibody level with an ELISA O.D of 0.291 and 0.232 one and two months post-immunisation.

Table 4.3.2: Humoral antibody level of fish injected and immersed with *Streptococcus* sp. formalin killed cells

Fish status	ELISA OD mean	S.D.	min	max	positive/negative cut-off
Immunised fish					
1m (i.p.)	0.291[a]	0.044	0.212	0.416	0.193
1m (bath)	0.121[c]	0.035	0.087	0.162	0.175
2m (i.p.)	0.232[b]	0.039	0.225	0.445	0.189
2m (bath)	0.132[c]	0.029	0.090	0.151	0.169
Control fish					
1m	0.105[c]	0.104	0.067	0.165	-
2m	0.111[c]	0.035	0.082	0.172	-

Values with the same superscript were not significantly different ($P < 0.05$).

4.3.4 Discussion:

Fish immersed in the streptococcal bacterin did not show any humoral antibody response to the antigen. It is probable that there is no antigen uptake following immersion of fish or even a low uptake can not elicit antibody production. This is consistent with the finding of Sakai *et al.* (1987) who did not find detectable agglutination antibody in rainbow trout following immersion in ß-haemolytic *Streptococcus* sp. bacterin.

Injection vaccination using 1 mg *Streptococcus* sp. cells showed moderate to marginal antibody production in fish. Sakai *et al.* (1987) also found low antibody titres after intraperitoneal injection of formalin-killed bacteria in rainbow trout. Low antigenicity of bacterin for fish is possibly one interpretation for the low antibody response. It is implied from the work of Kusuda and Takagi (1983) that toxoid vaccine is better than the bacterin.

4.4 Experiment III Evaluation of protection (streptococcosis)

4.4.1 Introduction and Aims:

A few studies have been carried out to evaluate the protectiveness of active immunisation against streptococcosis as described in section 4.1.9. The use of passive immunisation against streptococcosis has not yet been attempted. In this experiment, the aims were to immunise fish passively and actively and then challenge them at different intervals in order to evaluate the protectiveness of these vaccination methods.

4.4.2 Materials and Methods:

Antisera:

Anti-*Streptococcus* sp. antisera (ASA) were produced as is described in section 2.4. The ELISA antibody levels of sheep and rabbit were equal to O.D of 2.10 and 0.637 respectively at dilution of 1:128 and that of fish (ASA) was 0.301 at dilution of 1: 64. Fish were immunised with 0.1 ml 100 g^{-1} body weight.

Bacterin for active immunisation

Formalin killed *Streptococcus* sp. for injection and immersion vaccination of fish was prepared as described in section 2.2. Fish were immunised with the bacterins as described in section 4.3.2.

Fish:

Rainbow trout average weight 57 ± 6.6 g at the time of immunisation were kept in 4000 L freshwater temperature controlled tanks (at 15°C) in the trout room. Lightly anaesthetised fish were tagged individually and treated by i.p. injection of 0.1 ml 100^{-1} sheep, rabbit, fish ASA or 1 mg (dry weight) of bacterin (active immunisation) and maintained in two 4000 L temperature controlled tanks (15°C) (Table 4.4.1). Control fish did not receive any treatment but were only tagged at the time of immunisation.

Virulent *Streptococcus* sp. with LD_{70} for challenge:

Fish were challenged with the virulent bacteria as described in section 2.10.2. In this experimental challenge fish were inoculated with 32-43 cfu 0.1 ml^{-1} (LD_{75}), 55-65 cfu 0.1 ml^{-1} (LD_{85}) and 4-7 cfu 0.1 ml^{-1} ($LD_{62.5}$) of live *Streptococcus* sp. cells at one month, two months and three months challenges respectively.

Table 4.4.1: Experimental design for challenge of passively and actively immunised fish with experimental infection of *Streptococcus* sp.

Treatment	Route of administration of antisera	Number of fish challenged(in two replicates)		
		1m	2m	3m
Sheep ASA	i.p.	24	24	24
Rabbit ASA	i.p.	24	24	24
Fish ASA	i.p.	24	24	24
Immersion vaccinated	immersion	24	24	24
Injected vaccinated(1 mg)	i.p.	24	24	24
Control (Untreated tagged)	-	24	24	24
			Total No. of fish= 432	

ASA= anti-streptococcal antibodies

Challenge:

All immunised and control fish were challenged i.p. (in the ventral midline immediately anterior to the pelvic fins) with 0.1 ml of the virulent bacteria after anaesthetising with benzocaine. Protection levels in actively and passively immunised fish were expressed in terms of relative per cent survival (RPS).

One, two or three months after passive immunisation, the allocated number of fish for each time were challenged intraperitoneally. The temperature was maintained at 20°C during the challenge.

The mortality percent (mean) in all actively and passively immunised groups including challenged control were calculated. RPS which showed the immunity status of the fish was obtained from the mortalities in different groups and controls and then compared as described previously.

4.4.3 **Results:**

One-month challenge:

The challenge was conducted so that a minimum of 70% of the non-immune fish died. Results of protection level of immunised fish after one month of immunisation is shown in Table 4.4.2.

Table 4.4.2: Mortality and RPS after challenge (with virulent *Streptococcus* sp., LD_{75}) of rainbow trout immunised passively with sheep, rabbit and fish ASA and actively immunised (by immersion and injection) at one month p.i. as compared to controls

Treatment	Number of mortalities caused by *Streptococcus* sp.*			
	Replicate 1	Replicate 2	Combined Replicates	RPS± S.E.
Sheep ASA	0/12	2/12	2/24	88.8 ± 11.10
Rabbit ASA	4/12	5/12	9/24	50.0 ± 5.56
Fish ASA	8/12	10/12	18/24	0.0 ± 11.10
Active immunisation (i.p.)	1/12	1/12	2/24	88.8 ± 0.0
Active immunisation (bath)	7/12	9/12	16/24	11.1 ± 11.33
Controls Challenged non-immune	8/12	10/12	18/24	0.0 ± 11.11

* Experimental challenge LD_{75}= 32-42 cfu 0.1^{-1} ml by i.p injection.

Regarding protection obtained from passive immunisation, the highest RPS (88.8%) was observed in those fish immunised by sheep ASA one month post-immunisation. It was 50% in the case of rabbit ASA (not significant from that of sheep ASA, $P< 0.001$) and 0% in the case fish ASA.

With respect to the results of RPS obtained following injection and immersion immunisation (88.88% and 11.10% respectively) and their comparisons with that of sheep ASA, it is obvious that RPS conferred by sheep ASA is similar to that of the injection immunisation.

No fish pathogens were detected from the survivors of the treated groups (kidney and brain were sampled and cultured on BHI and BA). One of the survivors from the control group was culture-positive 14 days post-challenge. The remaining survivors from the control group were negative in culture from kidney and brain.

Two-month challenge:

The allocated fish for two-months challenge were challenged i.p. with 55-65 cfu live *Streptococcus* sp. (Table 4.4.3).

Table 4.4.3: Mortality and RPS after challenge (with virulent *Streptococcus* sp., LD_{85}) of rainbow trout immunised passively with sheep, rabbit and fish ASA and actively immunised (by immersion and injection) at two months p.i. as compared to controls

Treatment	Number of mortalities caused by *Streptococcus* sp*			
	Replicate 1	Replicate 2	Combined Replicates.	RPS± S.E.
Sheep ASA	8/12	6/12	14/24	33.3 ± 9.52
Rabbit ASA	10/12	9/12	19/24	6.8 ± 4.76
Fish ASA	11/12	8/12	19/24	6.8 ± 14.27
Active immunisation (i.p.)	7/12	6/12	13/24	38.1 ± 4.76
Active immunisation (bath)	9/12	11/12	20/24	4.7 ± 9.51
Control Challenged non-immune	10/12	11/12	21/24	0.0 ± 4.89

* Experimental challenge LD_{85}= 55-65 cfu 0.1^{-1} ml by i.p injection.

Protection level in this trial ranged from RPS 38.1% (active immunisation with i.p. injection of cells) to RPS of minimum 6.8% (related to rabbit and fish ASA). The highest rates obtained from injection immunised fish (RPS of 38.1%) and sheep ASA (RPS of 33.3%). There were no significant differences between the protection level of fish passively immunised with sheep ASA and fish immunised actively with formalin killed cells. However, protection rate for dip immunised group was very low (RPS= 4.7%). None of the protection obtained from passive and active immunisation in this trial could protect fish significantly after two months post-immunisation when compared with control fish ($P< 0.05$).

When comparing the protection level of fish at one and two months, the second challenge at 2 month post-vaccination showed that the protection declined markedly in passively immunised fish with sheep, rabbit and fish having RPS values declining from 88.8% to 33.3%, 50% to 6.8%, and 0 to 6.8% respectively. That of the actively i.p. immunised fish after 2 months also declined markedly.

Two fish from dip vaccination and one fish from sheep ASA injected survivors were culture-positive (kidney and brain were cultured on BHI and BA). The control groups which had not been immunised showed one positive fish in culture both from the kidney and the brain. The remaining survivors from different groups were culture negative 14 days post-challenge.

Three-month challenge:

Results of challenge of the immunised fish allocated for three months post-immunisation challenge (challenged i.p. with 4-7 cfu *Streptococcus* sp.) are shown in Table 4.4.4.

Table 4.4.4: Mortality and RPS after challenge (with virulent *Streptococcus sp.*, $LD_{62.5}$) of rainbow trout passively immunised with sheep, rabbit and fish ASA and actively immunised (by immersion and injection) at three months p.i. as compared to controls

Treatment	Number of mortalities caused by *Streptococcus* sp*			
	Replicate 1	Replicate 2	Combined Replicates.	RPS± S.E.
Sheep ASA	6/12	7/12	13/24	13.3 ± 6.66
Rabbit ASA	6/12	9/11	15/23	0.0 ± 20.0
Fish ASA	8/12	6/12	14/24	6.6 ± 13.32
Active immunisation (i.p.)	5/10	3/10	8/20	36.0 ± 16.0
Active immunisation (bath)	7/12	8/11	15/23	0.0 ± 11.51
Control Challenged non-immune	6/12	9/12	15/24	0.0 ± 20.0

* Experimental challenge $LD_{62.5}$=4-7 cfu 0.1 ml^{-1} by i.p. injection

Results indicated that the protection conferred by i.p. administration of immune sera after three months, were marginally effective to almost ineffective (protection ranged from RPS of 13.34% to RPS of 0%).

Protection rate was highest (RPS) in the group injected with 1 mg (dry weight) of formalin killed *Streptococcus* sp but significantly higher than those of other groups. Nonetheless, it was only moderately protective (RPS= 36%).

One fish from sheep ASA treated survivors was detected culture-positive (kidney and brain were sampled cultured on BHI and BA). The remaining survivors from different groups including control group, were culture negative up to 14 days post-challenge.

The relative per cent survival of fish immunised passively with ASA and actively with streptococcal bacterins after challenge are also shown in Figure 4.4.

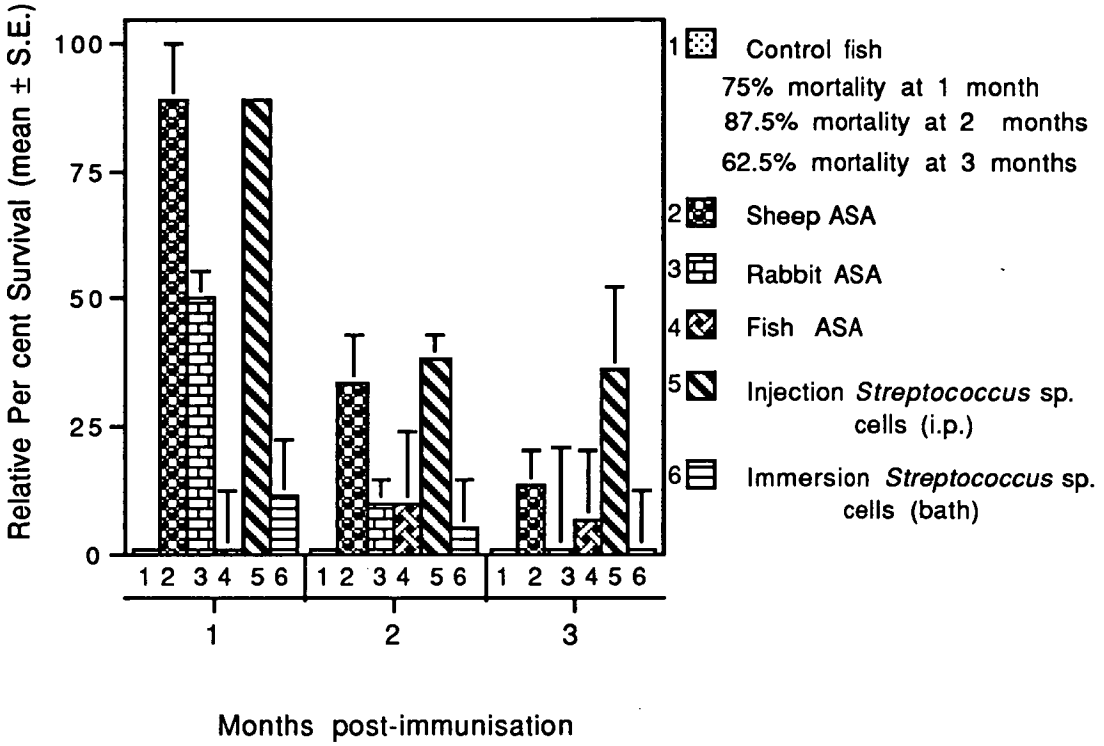


Figure 4.4: Survival rate of fish passively and actively immunised against *Streptococcus* sp. when challenge

4.4.4 Discussion:

The control fish showed 75, 85 and 62.5% mortality after one, two and three months respectively. In contrast, fish immunised with sheep and rabbit immune sera and injected bacterin showed appreciable protection (Figure 4.4). Strongest protection occurred in fish immunised with sheep ASA serum. Sheep ASA by injection conferred significantly higher protection than that of immersion. It seems that the level of immunoglobulins in 0.1 ml of sheep and rabbit antisera are high enough to give fish a good protection at one month or more after injection against the disease. These Ig are possibly distributed and stored in different organs following immunisation of fish. Interestingly, fish do not respond against them anaphylactically. Strong challenge (85% mortality) during trials decreased the protection rate (RPS) significantly. Nevertheless, higher protection might not have been obtained even if low number of bacteria were used. In this experiment, fish immunised with fish ASA, did not show

any protection. This is consistent with the results of ELISA (section 4.3.3) suggested low level of ASA in fish sera.

The results of this study is in agreement with the findings of Sakai *et al.* (1987) who vaccinated rainbow trout against ß-haemolytic streptococcal disease using formalin killed cells with and without Freund's complete adjuvants both intraperitoneally and by immersion. Their results showed 70% protection of fish following challenge with live bacteria. However, the results of immersion vaccination in this study contradict their report. This is probably because they used 3 min immersion and a 30 seconds immersion has been used in this study. It is possible however that strain variation between Japanese and Tasmanian isolates have their own uptake potential. In contrast with the findings of Sakai *et al.* (1987), the results reported here using immersion vaccination does not show such a high protection.

In agreement with the finding of Iida *et al.* (1981) who reported that intraperitoneal injection, was effective in yellowtail vaccinated against alpha-haemolytic *Streptococcus* sp., the results reported here indicate that Tasmanian -haemolytic *Streptococcus* sp. bacterin delivered by intraperitoneal injection to rainbow trout is effective immunogen against streptococcosis.

Anti-*Streptococcus* sp. agglutinating antibody titres were low (measured by ELISA) in i.p. injected fish (section 2.8). This is consistent with the low agglutination antibody following immersion in the bacterin and oral immunisation described by Iida *et al.* (1981). These antigens are probably not detectably immunogenic in rainbow trout. It is probable that *Streptococcus* sp. antigens were more immunogenic in sheep and rabbits.

With regard to the results of injection vaccination, it seems likely therefore that despite poor immunogenicity of the bacterin in the host, significant protection is achieved by actively immunising of fish which could be explained by inducing the humoral and cell-mediated responses and possibly non-specific responses of fish by the immunogen components of the cells even though we can not detect them by agglutination or ELISA. Nevertheless, it's proper mechanism needs clarification. Kusuda and Takagi (1983) investigated antibody production against α- haemolytic *Streptococcus* sp. in naturally infected yellowtail and found high titres were induced by the bacterium. Vaccination of fish using the more immunogenic antigen than simply whole cells is proposed (Sakai *et al.*, 1987). A RPS of 88.8% post-vaccination for the injection group was found in this investigation which is being reporting for the first time.

These observations suggest that protective immunity was mainly due to the action of specific Ig in strong challenges (i.p.) and concurrently exposure of fish to a 40% natural transmission (section 2.11.2). However the collaboration of cell-mediated and non-specific immunities have probably been an essential factor to eliminate the infection. It needs further work to elucidate the role of these components.

The mechanism of protection provided by sheep and rabbit antisera to *Streptococcus sp.* antigens is presumably simply the neutralising of the biological effects of toxins, since mammalian antibodies are incapable of activating rainbow trout complement (Sakai, 1981b) so this can not kill bacteria or act as opsonins. Furthermore, the level of protection and its duration in passively immunised fish will depend upon the rate at which the rabbit antibodies are complexed by the bacterial antigens released in the fish. This may explain the pattern of the results reported in the work of Ellis *et al.* (1988) where the Ranti 048 antiserum (antiserum to MT048, a less virulent protease-proficient *A.. salmonicida* cells and ECP) produced a higher level of protection and slower mortality rate in fish challenged with *A. salmonicida* (strain MT028) since there would be relatively more antiprotease antibody available to neutralise the smaller quantities of protease released from the more virulent protease-deficient MT028 as compared with the less virulent protease-proficient MT048.

It is probable that some of the protection resulted from non-specific immunity conferred by the injection of antisera irrespective of specific antibody as such was the finding of Ellis *et al.* (1988) that the control rabbit serum (rabbit injected with Freund's complete adjuvants alone) resulted in some degree of protection compared with saline-injected fish. The control rabbit serum possessed no detectable antibodies to *A. Salmonicida* antigens in any of the assays used. The protective effect is, therefore, likely to be due to non-specific effects possibly relating to toxin neutralisation or macrophage activation via, for example, interleukins. However, in an experiment reported earlier (experiment VII), it was shown that non-specific factors could not confer immunity to fish against vibriosis (the rabbit was not injected and immunised by any adjuvant or bacterin). Non-specific defense mechanisms such as phagocytosis and complement, lysozyme and transferrin activities have been reported in fish (Fletcher and White, 1976).

Despite the low challenge level ($LD_{62.5}$) after three months, marginal protection occurred in fish immunised with injection vaccination (RPS of 36%). The mechanism by which immersion vaccination confers protection appears to be associated with cell mediated and non-specific immunity. However, long term protection (after three months and more) and its relation with non-specific immune response and other mechanisms involved are still unclear and need to be elucidated.

CHAPTER 5.0

PARAMOEBIASIS, PASSIVE AND ACTIVE IMMUNISATION AND EVALUATION OF PROTECTION

5.1 Introduction/Literature review:

Amoebic gill disease (AGD) was first diagnosed in sea farmed salmonids on the south east coast of Tasmania in the summer of 1984-1985 (Munday, 1985). During this period, mortalities of up to 10% per week occurred during the second wave of infection in Atlantic salmon and rainbow trout (Foster and Percival, 1988a). There can be up to three outbreaks of AGD infection during the months of elevated water temperature when there is an absence of freshwater flushing from river outlets. Present control measures rely on treatment with freshwater which is difficult and costly. Therefore, immunoprophylaxis is an attractive alternative. Observations have suggested that previously-exposed salmonids are relatively resistant to reinfection i.e. immunity develops. The possibility of an immune response to *Paramoeba* sp. (PA) in recovered fish is promising but requires verification.

5.1.1 *Paramoeba* sp., the causative agent of amoebic gill disease (AGD):

The amoebae are present in large numbers, loosely attached to the surface of gill filaments and floating free in fresh preparations of the gill arch. The amoeba has a diameter of 20-30 μm with up to 50 digitate processes (pseudopodia) extending from the surface. They are motile and produce wide, rounded to conoid, clear pseudopods if left undisturbed on a slide (Langdon, 1990). Out of water this form retracts to a rounded granular body of 20 μm diameter. A locomotive form attached to the slide shows wide diaphanous pseudopodia.

The amoeba found associated with damaged fish gill tissue was classified as a *Paramoeba* species in 1987 (Foster and Percival 1988b) due to the presence of parasomes [(also known as Nebenkörper (Perkins and Castagna, 1971) and Nebenkern (Hollande, 1980)]. These intracellular inclusions of the *Paramoeba* are thought to be related to the presentation of a kinetoplastid flagellates (Hollande, 1980). Such parasomes contain DNA and can be demonstrated by Feulgen's, Heidenhain's iron haematoxylin and DAPI (fluorochrome 4',6-diamino-2-phenylindole) stains (Rogerson, 1988).

From pathogenicity trials conducted in 1993 it was noted that the cultured *Paramoeba* could not elicit the disease in fish (Howard *et al.*, 1993b).

Roubal *et al.* (1989) suggest that *Paramoeba* are primary pathogens capable at least of mechanically damaging gill epithelium. Their pseudopodia penetrate between epithelial cells and deform their surface. Abundant *Paramoeba* were invariably associated with extensive gill hyperplasia, and there was no evidence that the necrosis was caused by bacteria prior to the onset of *Paramoeba* infection. Initially, *Paramoeba*

sp. were found only on salmonids, but subsequently they were detected at an unpredictable frequency on a range of native marine fish (Munday *et al.*, 1990).

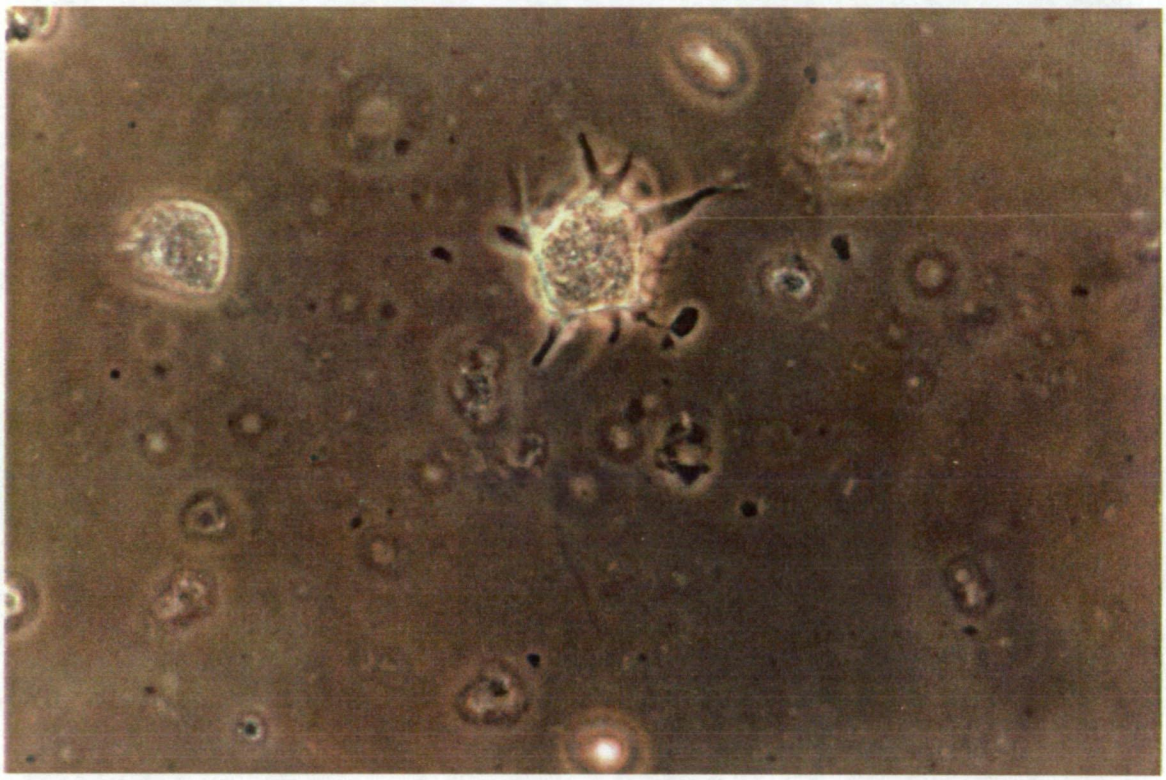


Plate 5.1.1: Fresh wet mount of the gills, showing *Paramoeba* sp.; 1000 X

Paramoeba is cultured on a variety of laboratory media e.g. on a bacterial 'lawn' of *Pseudomonas maltophilia* grown on malt and yeast extract seawater agar. One of the major problems facing the culture of pathogenic amoeba is the possibility of loss of virulence of the organism with time removed from the host. For example, Vincent and Neal (1960) found a gradual decrease in virulence occurred from polyxenic culture, but pathogenicity could be restored by passaging the organism (*Entamoeba histolytica*) through host cells.

5.1.2 **Epidemiology**

In Tasmania, disease associated with *Paramoeba* usually occurs in the first summer of sea cage farming of salmonids following their transfer from the freshwater hatchery (Langdon, 1990). The amoebae become apparent in low numbers on the gills two months after the introduction of fish to the sea (Foster and Percival 1988a). Increased severity of the disease occurs with the stress of elevated water temperatures (greater than 15-17°C) and factors such as poor hygiene, crowding, and decreased water exchange through biofouling on nets (Langdon, 1990; Munday *et al.*, 1990). However, at low stocking density, fish may still succumb to the disease (Foster and Percival, 1988b).

Elevated water temperatures probably act to decrease generation time and lead to a higher population size of amoebae (Kent *et al.*, 1988). This is consistent with the optimum temperature for *in vitro* culture being ~20°C (Howard and Carson 1991). Increasing water temperature leads to a decrease in levels of dissolved oxygen in the water. As a result fish oxygen demand increases through increased metabolic activity. Roubal *et al.*(1989) also suggested that the temperature stress on the fish may favour the increase in the parasite population.

There are still many unknowns relating to the sequence of events proceeding the outbreak of the disease. However, the cause of mortality in salmonids (which can be as high as 2% per day) appears to be respiratory and/ or osmoregulatory failure associated with a branchitis and respiratory stress (i.e. flared operculum and sluggish movement) (Munday *et al.*, 1990).

AGD occurs in both rainbow trout and Atlantic salmon in Tasmania (Munday *et al.*, 1990) coho salmon in Washington State and California, USA (Kent *et al.*, 1988) chinook salmon in New Zealand (C. Anderson, pers. comm.; Howard and Carson, 1993a) and Atlantic salmon and rainbow and brown trout in France (B. L. Munday, pers. comm.) farmed in the marine environment. The disease appears to be more severe in fish up to 2 kg body weight (young fish in their first season in the sea) (Munday *et al.*, 1990).

5.1. 3 **Clinical signs and pathology:**

The infected fish are sluggish and swim with open opercula (Munday *et al.*, 1990). They usually swim at the surface. Mortality is variable, reaching 2% per day and up to 50% total in untreated cages (Munday *et al.*, 1990). Subclinically and clinically affected fish have elevated plasma sodium levels (Langdon, 1990).

The outstanding pathological change is the presence of excessive mucus on the gills. In rainbow trout, this mucoid branchitis is relatively diffuse, but with Atlantic salmon, the lesions tend to be patchy (Munday *et al*, 1990). Epithelial hyperplasia involving the secondary lamellae, numerous active secreting mucus cells (Munday *et al.*, 1990), slightly thicker tips of the primary lamellae, prominent chloride cells and fusion of lamellae (Howard and Carson, 1992) are histological features of the disease. Histopathological changes in visceral organs are not associated with amoebae infestation (Kent *et al.*, 1988).

Plate 5.1.2: Signs of amoebic gill disease (AGD), showing typical white patches on gills

5.1.4 **Diagnosis**

The swimming behaviour of fish and the presence of white patches on the gills provide a tentative diagnosis. Fresh wet preparations of infected gills gently prepared in ambient seawater and observation of the *Paramoeba*, histo-pathological changes to gills (Munday *et al.,* 1990) as well as immunofluorescent antibody test (Howard and Carson, 1993a, 1994) are used to confirm the tentative diagnosis.

5.1.5 **Treatment**

The only effective treatment to date has been the use of freshwater baths (Munday *et al.*, 1990).

5.1.6 **Immunity against AGD:**

Observations have suggested that previously-exposed salmonids are relatively resistant to reinfection i.e. immunity develops. However, in some instances, rainbow trout and Atlantic Salmon may show disease again a few months after treatment (Langdon, 1990). The possibility of an immune response to *Paramoeba* in recovered fish is promising, but requires verification. Fish immune response to *Paramoeba* was speculated since both epidemiological and histological features of the disease suggested that there is development of immunity ,"judicious use of freshwater baths can be employed to produce a degree of natural vaccination"(Munday *et al.,* 1990).

5.2 Experiment I Passive and active immunisation against AGD

5.2.1 Introduction and Aims:

The following experiment was conducted to evaluate the longevity of passively transferred sheep anti-*Paramoeba* sp. antisera and to investigate humoral responses of fish to AGD antigens, . Aims were as follows:

-To produce a large quantity of hyperimmune serum against *Paramoeba* sp. in sheep.

-To develop a diagnostic assay to detect and compare fish antibody production to the causative agent of AGD.

-To determine how long sheep antiserum persists after injection into fish.

5.2.2 Materials and Methods.

Paramoeba:

Paramoeba sp. (PA-016) was obtained from the Fish Health Unit, Mt. Pleasant Laboratories, Department of Primary Industry and Fisheries, Launceston. It was maintained on a bacterial 'lawn' of *Pseudomonas maltophilia* grown on malt and yeast extract seawater agar as described in section 2.2.1.

Paramoeba culture:

Most amoebae grow well on a solid phase substrate such as agar, particularly with an overlay of liquid. Malt extract, yeast extract 75% seawater agar is commonly used and recommended as a substrate for amoebae culture (Kalinina and Page, 1992; Howard *et al.*, 1993b). Martin (1985) compared suspension culture to solid substrate culture and found that *Paramoeba pemaquidensis* did not increase in number in suspension culture. This is consistent with findings with the causative agent of AGD (Howard and Carson 1991).

Paramoeba was cultured according to the method described by Howard and Carson (1993b). Briefly, a medium consisted of malt and yeast extract seawater agar, comprising of 750 ml 0.2 µm filtered seawater, 250 ml distilled water, 0.1 g malt extract, 0.1g yeast extract and 30 g agar (Oxoid No. 1) was prepared (steam autoclaved at 121°C for 20 minutes). Whilst the agar was cooling, at a temperature approximating 60°C, 1 ml of 1% pimaricin (Sigma) L^{-1} as antifungal agent was added. The agar was then placed in 23 cm^2 bioassay dishes (Nunc, Denmark).

Plates were left to solidify overnight at room temperature, and were seeded with 2 ml of a *P. maltophilia* suspension (~1.5 x 10^8 cells ml^{-1}) in 0.2 µm filtered autoclaved seawater. The plates were then inoculated with 5 ml of amoeba suspension (~800 amoebae cells) extracted from a 2-6 week old established culture 24 hours after the addition of bacteria .

Amoebae culture plates were sealed with cellophane tape to prevent evaporation and maintained in an incubator at 20°C. Subculturing was conducted every 4-6 weeks. Finally a dense *Paramoeba* suspension was harvested from the plates.

Pseudomonas culture:

Pure cultures of *P. maltophilia* obtained from Mt. Pleasant Animal Health Laboratories were grown in Oxoid nutrient broth No. 2, at 37°C. *P. maltophilia* was extracted from the broth after 48 hours incubation by centrifugation. The cells were subsequently washed with PBS, aliquoted and stored at 4°C. Fresh cultures of this bacterium were maintained throughout the experimental period by regular subculturing on sheep blood agar plates.

Preparation of amoebic antigen:

In order to produce antisera in sheep and a rabbit against *Paramoeba* sp. the following protocol was established.

-Live *Paramoeba* (PA) from 3 plates were harvested with 3 ml of 0.2µm filtered seawater for each plate using bent pasteur pipettes 'hockey sticks'. A total of 8 ml was harvested.

-The suspension was divided into 4 tubes and then washed (4-5 volumes) in sterile filtered seawater by 3 centrifugation steps of 10 minutes at 500 g. Each time the supernatant was discarded (it contained most of the bacteria) and the pellet was resuspended again in the sterile seawater by vortexing.

-The efficiency of bacterial removal was tested by direct microscopic examination (wet smear; 400 times phase contrast) and by Gram stain. Still quite a few bacteria surrounded the *Paramoeba* and some within the amoebae. However, in each field 4-5 live *Paramoeba* existed in the final suspension.

- A dense solution of washed cells were then frozen and thawed several times and sonicated for 3 minutes. A 3 ml suspension was further inactivated (0.5% formalin), left at 4°C and finally 50% v v^{-1} FCA was added and the solution was homogenised.

- Protein content of the sonicated antigen was determined using a Lancer Microprotein rapid stat diagnostic kit. This test kit utilised Coomassie Brilliant Blue to attain a quantitative colorimetric determination of total protein.

Preparation of crude antigen (wild PA) from diseased fish:

Mucus from the gills of 40 severely infected Atlantic salmon was collected by scraping the gills. Normal saline was added to the suspension and homogenised. The suspension was filtered through glass wool and inactivated with 0.5% v v^{-1} formalin overnight. This crude vaccine was tested for sterility by culturing on blood agar, homogenised with 50% v v^{-1} FCA and kept frozen (-20°C) until used.

Animals

Sheep:

Two sheep were injected subcutaneously in the cervical area with a total of 3 ml of antigen [1.5 ml PA solution (containing approximately 1 mg protein) and 1.5 ml FCA per dose per sheep] . Four weeks later, sheep were boosted with the same amount of antigen, but in 50% v v^{-1} Freund's incomplete adjuvant. At the end of six weeks a large volume of blood was collected and the antiserum was collected and frozen at -20°C until used as described in section 2.4. The sheep response to PA was tested by both ELISA and immunofluorescent antibody assays.

Rabbit:

Rabbit antiserum was prepared as described by Howard and Carson (1991) with slight modification. Briefly, sonicated antigen obtained from 300,000 to 3 million *Paramoeba* was injected into the marginal ear vein of a New Zealand white rabbit by several injections (total inoculum = 1 mg protein) at 3-day intervals for 3 weeks. Antiserum was collected 21 days post-inoculation by complete exsanguination of the rabbit. Rabbit antiserum was tested by immunofluorescent antibody test for its activity and stored at -20°C for later use.

Fish:

1- In order to study the effect of the sonicated antigen (prepared from cultured *Paramoeba* sp.) in FCA on the humoral response of rainbow trout, twenty five fish (mean weight 127 g) from the Aquaculture Key Centre (AKC) were immunised with 1 mg (protein concentration) of the sonicated antigen.

2- In order to study the effect of the crude antigen in FCA on the humoral responses of Atlantic salmon, fifteen fish (mean weight 62 g) from AKC were used.

3- For determination of the rate of clearance of sheep APA from fish sera, 100 Atlantic salmon, 50 fish from AKC (mean weight 62± 8.2) and 50 fish (mean weight 57± 7.0) from Salmon Enterprises of Tasmania (SALTAS) were used according to the experimental design given in Table 5.2.1.

Fish were maintained in two 4000 L freshwater temperature control tanks at 15°C in recirculating biofilter systems at the Aquaculture Key Centre and fed daily with commercial trout pellets twice daily. Fish maintenance was the same as described in section 2.9.

4- Control fish: Twenty five fish each of SALTAS and AKC origin were bled from the caudal vessels prior to the commencement of the experiment. Sera were harvested as described in section 2.4 and used as controls in the ELISA.

Table 5.2.1: Experimental design for monitoring the rate of clearance of sheep APA from fish sera

Groups	No. of fish*	Treatment i.p. injection	Blood collection		
			1 week	4 weeks	8 weeks
Control	40	PBS	20	20	20
Hyper-immune serum	60	0.1 ml sheep antiserum	40	40	40
		Total number of fish (in two replicates)= 100			

Intraperitoneal injections

1- Control group: Forty fish (20 from SALTAS and 20 from the Key Centre) were tagged, injected with PBS and kept in the tanks.

2- Passive immunisation with anti-*Paramoeba* sp. hyperimmune serum prepared in sheep.

Thirty fish (15 from SALTAS and 15 from the Key Centre) for replicate 1 in one tank and 30 fish (15 from SALTAS and 15 from the Key Centre) for replicate 2 were allocated for this group. Sheep APA antiserum was used for intraperitoneal injection of fish. Fish were injected (1 ml syringes, 25 G x 19 mm needles) with the hyperimmune serum at a rate of 0.1 ml $100g^{-1}$ fish body weight. The rate of elimination of sheep anti-*Paramoeba* antiserum in fish body was monitored at one week, 4 weeks and 8 weeks post-immunisation.

Immunisation of fish with crude antigen:

Fifteen Atlantic salmon were injected with 0.5 ml of the crude antigen in FCA (wild *Paramoeba* sp.) in order to monitor their humoral response to this antigen. Fish were kept in temperature controlled (15°C) tanks and bled 6 weeks post-immunisation

Immunological assays for detection of anti-*Paramoeba* sp.:

An indirect fluorescent antibody test and an specific ELISA were employed to determine the antibody response of rabbit and sheep to *Paramoeba*.

Immunofluorescent antibody test (IFAT):

The immunofluorescent antibody test was employed as described by Howard and Carson (1991) . Briefly, *Paramoeba* smears were prepared from a culture plate and allowed to air dry. Smears were then fixed with 70% ethanol and air dried. Rabbit or sheep anti-*Paramoeba* antiserum (primary antibody), diluted to the appropriate

concentration in PBS was added. Slides were incubated for 60 minutes at 37°C in a moist chamber. They were rinsed for 5 minutes in PBS (repeated 3 times) and wiped dry. The secondary antibody, goat anti-rabbit or rabbit anti-sheep antiserum fluorescein isothiocyanate conjugated antibody (FITC) diluted at an optimal concentration in PBS was then applied. Slides were left to incubate for 60 minutes in a moist chamber. Slides were washed again in PBS and mounted in glycerine bicarbonate buffer, pH 9. Slides were observed by fluorescent microscopy for the presence of surface fluorescence of the *Paramoeba sp.* (Plate 5.2).

ELISA:

The ELISA used for detecting of antibodies against *Paramoeba* sp. was a modification of the ELISA described by (Bryant *et al.*, 1994). The ELISAs employed in the detection of both sheep APA (by sheep ELISA) and fish APA (by fish ELISA) were as described in section 2.6.5.

5.2.3 **Results**

Immunofluorescent antibody test:

The results of the IFAT on sheep anti-*Paramoeba* sp. were positive. Dilutions of 1: 50, 1: 100 and 1: 250 were used from which a dilution of 1: 100 was optimal.

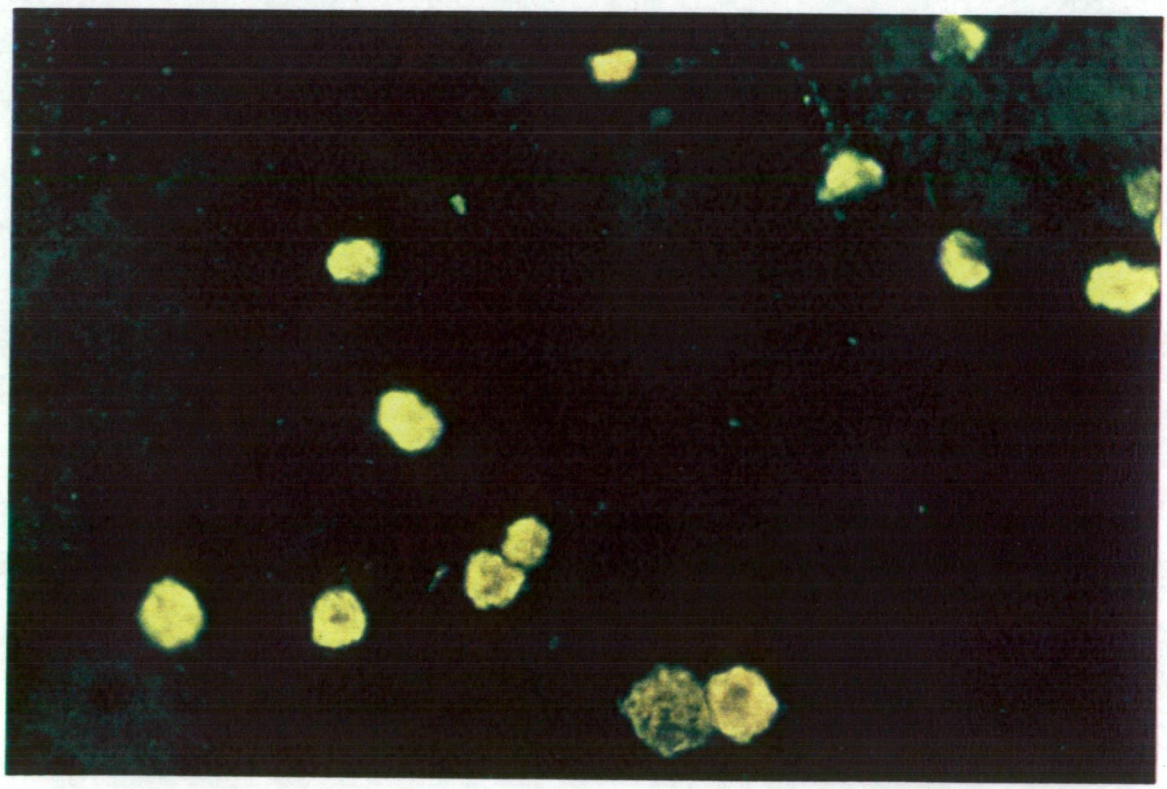


Plate 5.2: Fluorescent antibody test of sheep anti-*Paramoeba* antibodies, showing an intense yellow colour *Paramoeba* sp. cells; 1000 X (photo provided by courtesy of Ms Teresa Howard)

ELISA:

Active immunisation: The results of ELISA for immunised sheep (pooled sera of sheep), rabbit and fish (pooled sera of rainbow trout) are shown in Table 5.2.2. Sheep responded well to the sonicated antigen/ FCA . Rabbit showed a lower anti-*Paramoeba* antibodies level with O.D = 0.821 (sonicated antigen) and rainbow trout showed an O.D of no more than 0.421 when they were injected with the same antigens (sonicated PA + FCA). The ELISA O.Ds for control sera of sheep, rabbit and fish were previously shown in Figure 2.6.5.

Table 5.2.2: Humoral response of sheep, rabbit and fish (rainbow trout) to *Paramoeba* sp. vaccines

Dilution of sera	Sheep (pooled sera)		Rabbit		Fish (pooled sera)	
	Non-imm.	Imm.	Non-imm.	Imm.	Non-imm.	Imm.
1: 64	0.174	2.401	0.145	0.821	0.145	0.421
1: 128	0.156	2.341	0.153	0.701	0.146	0.354
1: 256	0.151	2.000	0.134	0.683	0.123	0.387
1: 512	0.145	1.921	0.131	0.434	0.132	0.254
1: 1024	0.133	1.653	0.099	0.241	0.111	0.183
1: 2048	0.123	1.324	0.098	0.171	0.095	0.101
1: 4096	0.121	1.245	0.074	0.149	0.119	0.098

Non-imm.= Non- immune serum Imm.= Immune anti-*Paramoeba* serum

Atlantic salmon: Atlantic salmon injected with formalin killed wild PA + FCA developed a significant antibody level at six weeks post-immunisation (mean ELISA O.D = 0.452). Only 40% of fish were seropositive and the rest of fish were seronegative (Table 5.2.3).

Table 5.2.3: Humoral response of Atlantic salmon to crude *Paramoeba* vaccine at 6 weeks post-immunisation

Immunised Atlantic salmon	No. of fish	Percentage of seropositive	ELISA O.D mean ± S.E.	Maximum	minimum
Wild PA/ FCA	15	40	0.452^{a}± 0.096	1.324	0.075
Control fish (AKC naive fish)	25	-	0.092^{b}±0.037	0.142	0.078

Means with the same superscript are not significantly different (P<0.05).

Passive immunisation: The rate of clearance of sheep APA from Atlantic salmon sera (mean ELISA O.D± S.E. of SALTAS and AKC fish) is shown in Figure 5.2.1. Sheep APA had a high level (O.D = 0.682± 0.053) at one week post-immunisation. These antibodies declined rapidly to 4 weeks then gradually to 8 weeks post-immunisation.

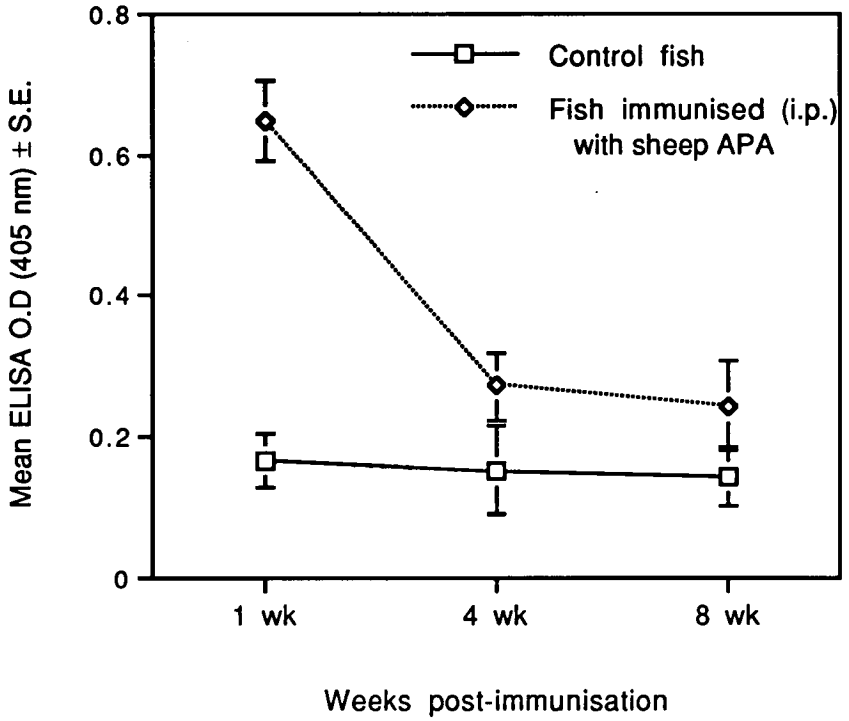


Figure 5.2.1: Rate of clearance of sheep APA from Atlantic salmon sera for up to 8 weeks post-immunisation from fish sera

5.5.4 Discussion:

Sheep, rabbit and fish developed humoral antibody when they were immunised with *Paramoeba* sp. vaccines (Table 5.2.2).

When sheep APA were injected intraperitoneally into Atlantic salmon to passively immunise fish, it was shown that these antibodies were long lasting (up to 8 weeks) (measured by sheep ELISA in fish sera). Therefore, the persistence of these antibodies can provide fish with passive immunity if the specific immunity proves to be protective.

5.3 Experiment II Evaluation of protection (paramoebiasis)

5.3.1 Introduction and Aims:

This experiment was conducted in order to evaluate the effectiveness of passive immunisation (passively transferred antisera into fish) and active immunisation against AGD. Aims were as follows:

-To demonstrate protection against AGD as a result of injecting the anti-*Paramoeba* antisera into fish and challenge the fish by cohabitation with AGD infected fish.

- To immunise fish actively with live and sonicated *Paramoeba* sp. and challenge them to investigate the effectiveness of this immunisation.

- To evaluate the efficacy of a crude vaccine (wild *Paramoeba* sp.) against the disease.

5.3.2 Materials and Methods

Preparation of antigens:

Antigens were prepared as described in section 5.2.2.

Preparation of sheep and rabbit anti-*Paramoeba* sp. sera:

Sheep and rabbit anti-*Paramoeba* sp. sera were prepared as described in section 5.2.2.

Preparation of crude (wild *Paramoeba* sp.) antigen from diseased fish:

This antigen was prepared as described in section 5.2.2.

Fish:

Trial 1- A total of 640 Atlantic salmon, 320 Atlantic salmon "smolt" (brought from Wayatinah; SALTAS freshwater operation, (mean weight 57±7.0 g) and 320 Atlantic salmon "smolt" from the Aquaculture Key Centre (mean weight 62±8.2 g, the same stock of fish used for experiment I section 5.2) were used for evaluation of protection following passive and active immunisation.

Fish were maintained in two 4000 L freshwater temperature control tanks at 16°C in recirculating biofilter systems at the Aquaculture Key Centre and fed daily with commercial trout pellets (Gibson) twice daily.

All fish used in this study were previously unexposed (naive) to paramoebiasis. The experimental design for protective value of hyperimmune sera and vaccination with different *Paramoeba* antigens is shown in Table 5.3.1. Forty fish (20 from SALTAS and 20 from AKC) for replicate 1 in one tank and 40 fish (20 from SALTAS and 20 from the Key Centre) for replicate 2 were allocated for each group.

Table 5.3.1: Experimental design for protection trial (paramoebiasis)

Groups	No. of fish*	Treatment i.p. injection	Exposure to fish infected with *Paramoeba* sp. 1st	2nd
Control	80	PBS	1 month	45 days
Hyper-immune serum	80	0.1 ml sheep antiserum	1 month	45 days
Hyper-immune serum	80	0.1 ml rabbit antiserum	1 month	45 days
ØLive PA injection	80	3800 live PA in 0.1 ml	1 month	45 days
ØSonicated PA in PBS	80	1 mg sonicated P.A antigen (protein content)	1 month	45 days
ØSonicated PA in PBS	80	10 mg sonicated P.A antigen (protein content)	1 month	45 days
ØSonicated PA in FCA	80	1 mg sonicated P.A antigen (protein content)	1 month	45 days
ØSonicated PA in FCA	80	10 mg sonicated P.A antigen (protein content)	1 month	45 days

Total number of fish (in two replicates)= 640

* In each group 40 Atlantic salmon from SALTAS and 40 fish from the Key Centre.
Ø From studies undertaken in collaboration with Ms Kirsten Rough, MAppSc candidate.

All fish were starved 12 hours before commencement of the experiment, then anaesthetised before tagging or/and any treatment.

Intraperitoneal injections:

1- Control group: Fish were injected intraperitoneally with 0.1 PBS as described in section 5.2.2.

2- Passive immunisation with sheep anti-*Paramoeba* sp. hyperimmune serum prepared in sheep and rabbit.

Sheep and rabbit antisera were used for intraperitoneal administration into fish. Fish were injected with the hyperimmune serum at a rate of 0.1 ml $100g^{-1}$ fish body weight as described in section 5.2.2.

3- Live *Paramoeba:* A pellet of *Paramoeba* was resuspended in the solution of 1% carbenicillin disodium salt (Sigma No. C-1389) in sterile seawater by vortexing gently and left to stand for 2 hours. The suspension was centrifuged (500 g) and the pellet was resuspended in sterile seawater. *Paramoeba* densities were determined by counting cells using a Neubauer haemocytometer, adjusted and suspended in 0.2 μm filtered sea water so that 0.1 ml of solution of 8×10^4 amoebae was injected intraperitoneally to each fish.

4- Sonicated *Paramoeba* with adjuvants: A cell count of washed *Paramoeba* was performed then an ultrasonic machine was used to disrupt the cell membrane. The suspension was filtered through an 0.2 μ filter and its protein content was determined (protein concentration 163 $\mu g\ ml^{-1}$). Different protein contents were prepared containing 1 mg and 10 mg with and without 50% v v^{-1} FCA. This solution was administered according to the experimental design by i.p. injection of 0.1 ml into each fish (Table 5.3.1).

Fish were randomly divided in two groups. All were weighed and individually tagged.

Fish were acclimatised to seawater 15 days before cohabitation (from 15 days post-immunisation) with the diseased fish. After one month post-immunisation fish were cohabited with 50 infected fish (25 for each tank) and water temperature was maintained at 18°C. Since *Paramoeba* sp. could not be detected up to two weeks after cohabitation another batch of infected large fish (5 fish) were placed in each tank. After one week, fish started to show clinical signs.

Mortalities were sampled for gill samples twice a day for histopathological survey. The temperature was maintained at 18°C.

Trial 2- Immunisation of Atlantic salmon with the crude vaccine:

Forty Atlantic salmon were injected i.p. with the crude antigen in the freshwater operation unit of SALTAS (Wayatinah). These fish were cohabited with infested fish with *Paramoeba* sp. at Dover (sea culture operation) two and a half months later for determination of the protection efficacy of the crude vaccine.

ELISA:

An ELISAs were run to monitor the humoral response of experimentally and naturally infected fish to *Paramoeba* sp. as described in section 5.2.2.

Immunisation (i.p. injection)	first challenge 1 month (unsuccessful)	2nd challenge	Blood collection
(Time 0)	30 days	45 days	60 days

320 fish (Atlantic salmon) ——▷ challenged ——▷ challenged ——▷ (B.C)
from SALTAS

320 fish (Atlantic salmon) ——▷ challenged ——▷ challenged ——▷ (B.C)
from the Aquaculture
Key Centre

40 fish (Atlantic salmon) ——▷ challenged 2.5 months post immunisation
from SALTAS
(Wayatinah)
(crude antigen)

52 fish (Atlantic salmon) ——▷ Blood collection for serology
2-year old fish (ELISA)
(naturally infected in sea cages)

40 fish (Atlantic salmon) ——▷ Blood collection for control sera
(Naive fish, control fish) in ELISA
(as described in section 5.2.2)

B.C= blood collection+ gill samples for histopathology

Figure 5.3.1: Flow diagram showing the time of immunisation, challenge and blood collection for serology of the experimental fish

Trial 3: Detection of local antibody (using ELISA) from mucus collected by perfusing gills

Gill mucus samples supplied by Ms. Venessa Findlay had been prepared as follows: Gills of 10 Atlantic salmon (mean weight 200g), experimentally infected with AGD for 3-6 weeks were perfused and the mucus was extracted according to the method described by Lumsden *et al.* (1993). Mucus was extracted in the same way from six naive fish. Sera were also collected from these fish.

5.3.3 Results

Protection:

Trial 1- No disease fish infested with *Paramoeba* was observed up to 15 days post-cohabitation (first exposure). It was assumed that the donor fish infestation had not persisted. Therefore, another stock of diseased fish was obtained and placed with the experimental fish at 45 days post-immunisation. From one week after cohabitation (second exposure) fish showed considerable mortality. *Paramoeba* were detected on the gills of almost all exposed fish. The temperature was decreased to 16°C. However, amoebic gill disease appeared in an outbreak form (morbidity= 100%) and rendered the evaluation of protection unreliable. Gill samples were collected to assess the infestation of the parasite quantitatively in order to attempt to draw a conclusion on protection. However, all fish showed severe gill patches, therefore the assessment was ineffective.

Trial 2- Forty Atlantic salmon vaccinated with the crude antigen (wild *Paramoeba* sp.) did not show any significant serological differences in comparison with those injected with laboratory cultured *Paramoeba* sp. These fish were challenged two and a half months post-immunisation in cohabitation with diseased fish. All the fish showed gross lesions of paramoebiasis and *Paramoeba* sp. was detected in almost all samples.

ELISA:

Two weeks after effective cohabitation exposure, survivors, which in fact were suffering severely from the disease, were euthanised for collection of gill samples for histopathology (in Davidson's fixative) and blood samples for serology. Antibody levels of fish as a result of the experimental infection after 2 weeks exposure to AGD are shown in Table 5.3.2.

Table 5.3.2: Humoral response of Atlantic salmon to the experimental infection with *Paramoeba* sp.

Groups of fish	No. of fish in two replicates	% seropositive	ELISA O.D of responders mean ± S.E.	Maximum	Minimum
Control (exposed)	32	50	0.298^{abd}±0.032	0.343	0.072
Sheep hyper-immune serum	35	45	0.345^{bd}±0.111	0.486	0.086
Rabbit hyper-immune serum	40	44	0.256^{a} ±0.057	0.329	0.088
Live P.A injection	26	53	0.341^{bd}±0.039	0.421	0.0113
Sonicated(1 mg) P.A in PBS	20	58	0.299^{abd}±0.067	0.411	0.142
Sonicated(10 mg) P.A in PBS	42	46	0.234^{a} ±0.042	0.501	0.123
Sonicated(1 mg) P.A in FCA	29	38	0.354^{bd} ±0.057	0.451	0.141
Sonicated(10 mg) P.A in FCA	37	49	0.365^{bd} ±0.023	0.467	0.086
Control fish (naive) (SALTAS and AKC fish, mean ELISA O.D)	50	-	0.101^{c} ±0.041	0.078	0.142

Means with the same superscript are not significantly different (P<0.05)

Results of histopathology of gills collected from mortalities are as follows: No *Paramoeba* or lesions were observed in the gill samples taken from the small number of mortalities after the first cohabitation indicating the absence of paramoebiasis. Gill samples collected seven to fourteen days after the second cohabitation (exposure to the second batch of infected fish), showed infestation with *Paramoeba* sp. and had mainly moderate to severe lesions.

Both naturally and experimentally infected fish had significantly higher antibody levels than control. There was no significant difference in antibody levels between these groups. However, the percentage of seropositive fish in the experimentally infected group was higher than in the naturally infected group (Table 5.3.3)

Trial 3- The ELISA results measuring the gill mucus antibody of the fish infected with *Paramoeba* sp. antigens were all negative. Eighteen per cent of these fish showed seropositive in the ELISA (Table 5.3.3).

Table 5.3.3: Comparative data for the immunological responses of fish exposed (or not exposed) to *Paramoeba* sp. by a variety of means

Naturally infected fish	No. of fish	Percentage of seropositive	ELISA O.D of responders mean ± S.E.	Maximum	minimum
Natural infection (prolonged)	52 (s)	35	0.354^{a}± 0.123	0.865	0.089
Natural infection (short)	8 (s)	25	0.192^{b}± 0.095	0.219	0.091
Severe experimental infection (2 weeks)	261*(s)	48	0.297^{a}±0.098	0.231	0.111
Moderate Experimental infection (3 weeks)	11**(s)	18	0.207^{a}± 0.112	0.231	0.108
	10 (m)	0	0.123^{b}± 0.096	0.132	0.106
Control fish (naive)	50 (s)	-	0.101^{b}±0.041	0.142	0.078
	6 (m)	-	0.116^{b}± 0.036	0.127	0.091

* collected sera from survivors of experimentally challenged fish (Table 5.3.1, 640 fish).
** collected sera from Trial 3. s= serum, m= mucus.
Means with the same superscript are not significantly different (P<0.05)

5.3.4 Discussion

Not all sera of presumed naturally-infected fish appeared to be seropositive (only 35% seropositive). Similarly, experimentally infected fish did not show a high response either, even though the percentage of seropositive in this group was higher (48%) than those naturally infected. This is consistent with the results of Grayson *et al.* (1991) who reported that Atlantic salmon can produce antibodies to artificially injected *Lepeophtheirus salmonis* (salmon louse) components, but naturally infected Atlantic salmon and rainbow trout did not similarly respond. It is clear that short term infection of naive fish can stimulate the humoral immune system. Perhaps long term infestation, particularly with small number of *Paramoeba* on the gill may produce immune tolerance. However, the observed unresponsiveness still remains unresolved.

No significant difference was observed between the antibody levels of fish naturally and experimentally infected fish with paramoebiasis. Thus, long exposure of fish does not seem to stimulate an enhanced immune response.

Despite the above limitations, fish ELISA results monitoring the antibody levels of fish exposed to *Paramoeba* antigens are promising for diagnostic purposes.

Cohabitation with presumed diseased fish did not produce the disease at the first attempt. However, a second batch of infected fish produced severe AGD. It is possible that the first batch did not carry many *Paramoeba* sp. on their gills possibly due to the development of immunity during the long course of infection.

Passively and actively immunised fish that were experimentally infected by cohabitation, showed humoral antibody response (measured by ELISA) to *Paramoeba* in a period of two weeks after exposure to *Paramoeba* sp. (Table 5.3.3). The humoral responses of these fish were significantly different from those of control fish (naive fish). This concludes that fish humoral antibody can be induced by a heavy AGD infection.

Some groups of the exposed fish which previously were immunised, showed significant differences with each other in antibody levels post-cohabitation (Table 5.3.2). This could be due to injection of antigen with adjuvant when they were immunised before cohabitation and the possible effect of adjuvant on their humoral responses. However this pattern was not consistent between groups of fish i.e live PA treated and sheep APA treated fish showed almost the same antibody level as sonicated P.A with FCA treated fish.

Crude antigen (wild *Paramoeba* sp.) harvested directly from infected fish was not superior antigenically from vaccines produced from cultured organisms. Fish immunised with this antigen died gradually when they were cohabited with infected fish two and a half months post-immunisation. Administration of this crude antigen with FCA into Atlantic salmon did not change the unresponsiveness status of non-responding fish (60% of vaccinated fish were seronegative). Since the most probable reason for lack of immunity when cultured *Paramoeba* was used, was thought to be the absence of virulence factors in the cultured organisms it is difficult to explain why a crude vaccine containing antigens from virulent organisms did not work. It was shown from findings of Lumsden *et al.* (1994) who injected (i.p. with 0.1 ml) and immersed rainbow trout (in a 1: 10 dilution) of acetone-killed *Flavobacterium branchiophilum* that the level of antibody to the bacterium was highest in the i.p. injected group. However, the percent cumulative mortality was 32.1, 11.7 or 45.3% in i.p. injected, immersed and control fish respectively. The i.p. injected group had the highest serum antibody levels, while bath exposure to the highest concentration of killed bacteria produced the highest gill-associated antibody levels. Therefore, gill associated antibodies might be the main protective value in immersion vaccinated fish especially against diseases which cause surface infections of gills.

Regarding the lack of protectiveness of mammalian sera in combating paramoebiasis, it should not be forgotten that in cohabitation, 100% mobidity was not expected. With such a high mortality level it is difficult to assess the protection efficacy.

The results of this study indicate that systemic antibody is possibly less important for protection from AGD if the organism infect gill tissues. Therefore, local immune response may play an important role in naturally infected fish. However, local antibody to PA could not be detected by using ELISA (Table 5.3.3).

The results obtained in this investigation have considerable potential value in relation to future immunisation trials, protection experiments and immunological assays. Further work is required to elucidate immunological aspects of AGD, possibly determination of antigenic components of *Paramoeba* sp. and genetic engineering may help to improve vaccines and/ or by manipulating the *Paramoeba* to deplete its virulence in near future to overcome such a problematic and important fish disease.

CHAPTER 6.0

GENERAL DISCUSSION AND CONCLUSIONS

Studies on the use of antisera to protect fish from vibriosis were pioneered by Harrell *et al.* (1975) and Viele *et al.* (1980) who reported considerable protection from immune fish and rabbit AVA against vibriosis; since then studies have been sporadic.

A great deal of effort has been expended to investigate the use of mammalian antisera against *A. salmonicida* with encouraging results. The most significant study on passive immunisation (of speckled trout and Atlantic salmon) is the report of Turgeon and Elazhary (1992) with an anti-*A. salmonicida* produced in mammals. However, the details of the antisera used in that report have not been released.

Preliminary work of Cipriano (1981) on transferring rabbit and salmonid fish anti-*A. salmonicida* Ig to rainbow trout and Atlantic salmon suggested that only fish Ig provided protective immunity . Rabbit antisera failed to provide protective immunity to fish.

The work of other people (Table 1.1) regarding the protectiveness of immune sera against fish diseases i.e furunculosis, edwardsiellosis, pseudotuberculosis and yersiniosis has shown variable results. However, some reports have been encouraging in relation to passive immunisation as a tool to control some fish diseases.

In the present study, the longevities of sheep and rabbit anti-*V. anguillarum* antisera up to 70 days were well demonstrated (using ELISA) and such persistence has not been reported before. Protection rate of these antisera at one month post-immunisation exceeded 90 and 86% survival rate in groups of fish vaccinated with sheep and rabbit AVA respectively. In another group that received fish AVA, the survival rate was somewhat less (RPS= 40%) but all groups were protected against acute vibriosis (Table 6.1).

Table 6.1: Relative per cent of survival after challenge in fish immunised passively and actively against vibriosis

Type of immunisation	1 month (LD_{75})	2 months (LD_{80})	3 months (LD_{73})
Sheep AVA	93.3%	25%	13.3%
Rabbit AVA	86.6%	18.7%	4.1%
Fish AVA	40%	12.5%	9%
(Active immunisation) Immersion	86.6%	93%	81.8%

Two months after immunisation, in a strong challenge (LD_{80}), the protection value of different sera declined, but that of immersion vaccinated group was still significant (RPS= 93%). Three months post-immunisation, only sheep AVA showed a marginal protection for fish (Table 6.1).

Immersion vaccinated fish showed marked protection. The survival rates in these experiments are consistent with other reports e.g. Håstein and Refsti (1986) reported only 3.5, 4.6, 6.7% mortality almost 3 months after active immunisation with immersion challenges of LD of 41, 54.6 and 61.7 per cent respectively. Amend and Johnson (1981), reported 0-7.5% mortality after one month of immersion vaccinated fish with immersion challenges of $1.7x10^5$ cfu ml^{-1} and $8.4x10^5$ cfu ml^{-1} respectively. Horne *et al.* (1982) reported 53% mortality at a 85 days post-vaccination challenge ($2x10^7$ viable cells for challenge) at a low water temperature(6-11°C).

With injected *Vibrio* antigens, agglutinating antibody titres are readily detected and titres are closely associated with protection (Harrell *et al.*, 1975). In contrast, vaccination by immersion or orally rarely result in demonstrable agglutinating titres. In this work these titres were monitored by ELISA (Figure 3.4.2). Nevertheless, despite the short period that antibodies could be detected in fish sera after immersion vaccination (1 month), a RPS of 81.8% was found 3 months post-immunisation. This explains why immersion vaccination of fish against vibriosis has been successful in practice all around the world. This could be due to the fact that non-specific responses or cell mediated immunity may have been activated with immersion vaccination. More research is required to elucidate this.

For passive immunisation, protection appeared to be associated with specific antibodies and not non-specific components i.e a) protection correlated with the level of specific antibody and as sheep AVA level declined in fish sera, the protection rate dropped, b) complement did not appear to be involved, because immune serum (complement inactivated, by 30 min at 56°C) was as protective as whole immune serum (with complement), c) non-immune sera (complement inactivated and with complement) injected i.p. into fish could not confer protection against vibriosis. A possible explanation for the difference of these results and results of Ellis *et al.* (1988) who showed that the animals supplying the non-immune sera in that study were injected with FCA whereas the one supplying the non-immune sera in this study were not so injected.

In this study, the feasibility of using passive immunisation with sheep and rabbit Ig as a protective measure and a medical treatment for vibriosis in cultured rainbow trout was encouraging. The maximum concentration of passive Ig in fish serum occurred during the first 24 hours after immunisation, but levels could be detected for more than three months (sheep and rabbit AVA) as measured by ELISA. Sheep and rabbit as well as fish hyperimmune sera made against *V.anguillarum* appears useful for passive protection of particularly valuable fish during those months when vibriosis is prevalent.

Furthermore, the induction of an active immune response in fish takes some time depending on the ambient water temperature (not less than a week or weeks from results of Atlantic salmon immunised and tested after one week in this study) and, therefore, fish vaccinated in transport tanks arriving at a site where an immediate epizootic is expected, will not be immune to the disease. Thus, passive immunisation is a real potential application especially as it has a relatively long therapeutic effect if it is used at strategic points.

Oral delivery of AVA to rainbow trout using protein-G purified antiserum prepared in sheep and purified antibodies conjugated to LTB as protein carrier was promising. As LTB by itself had no protective efficacy, it was presumed that protection was associated with specific antibodies bound to receptors at key sites such as the spleen, liver and kidney. It seems that the future of oral immunisation for fish is promising.

With respect to the results obtained from streptococcosis trials, it is obvious that this is the first time that such a work on aspects of passive immunisation of fish against the disease has been carried out.

In this study, the possibility of using passive immunisation with sheep and rabbit immunoglobulins as a protective measure and a medical treatment for streptococcosis in cultured rainbow trout was investigated. It is clear now that the use of antisera specific to the pathogenic organism, particularly the use of mammalian antisera is encouraging. Longevity of sheep ASA was not less than 75 days post-immunisation. Survival rates for 30 days challenge of fish immunised passively with sheep and rabbit ASA, were not less than 50% and sheep ASA protected fish moderately (RPS= 33.33%) up to 2 months post-immunisation (Table 6.2). Therefore, it can be concluded that passive immunisation of fish against streptococcosis with Ig is highly effective for at least one to two months after immunisation especially as Ig appears in fish sera within several hours after immunisation .

Table 6.2: Relative per cent of survival after challenge in fish immunised passively and actively against streptococcosis

Type of immunisation	1 month (LD_{75})	2 months (LD_{85})	3 months ($LD_{62.5}$)
Sheep ASA	88.8%	33.3%	13.3%
Rabbit ASA	50%	6.8%	0%
Fish ASA	0%	6.8%	6.6%
(Active immunisation)			
Immersion	11.1%	4.7%	0%
Injection	88.8%	38.1%	36%

Regarding injection immunisation with 1 mg formalin killed bacteria (dry weight), the survival rates in these experiments are consistent with other reports (Iida *et al.*, 1981) who reported that intraperitoneal injection, hyperosmotic infiltration and oral administration were effective in yellowtail vaccinated against alpha-haemolytic *Streptococcus* sp. Nevertheless, efficacy of oral vaccination using both dry and wet bacterin was prolonged for only two weeks after the last vaccination. The efficacy became more effective with an increase in frequency of oral vaccination. From this study, the effectiveness of active immunisation against streptococcosis with the agent of *Streptococcus* sp. (biovar 1), has been reported for the first time.

Vaccination as a control measure against streptococcosis has been recommended (Iida *et al.*, 1981, Kusuda and Takagi, 1983). Sakai *et al.* (1987) in a study vaccinated rainbow trout against ß-haemolytic streptococcal disease. They used formalin killed cells (bacterin) and toxoid with and without Freund's complete adjuvant intraperitoneally, by immersion and oral delivery of the bacterin. Their results showed 70% protection to fish following challenge with the live bacteria. Nevertheless, anti-*Streptococcus* sp. agglutination antibody titres were low in intraperitoneally immunised fish and not detectable agglutinating antibody was demonstrated following the immersion and oral immunisation route.

In comparison between injection vaccination and immersion administration the latter appeared the least effective although the level of protection may be acceptable in commercial operations when off set against the lack of stress and handling involved.

With regard to the *Paramoeba* immunisation and protection trial, passively and actively immunised fish that were experimentally infected by cohabitation, showed humoral antibody response (measured by ELISA) to *Paramoeba* in a period of two

weeks after exposure to *Paramoeba* sp. Crude antigen (wild *Paramoeba* sp.) harvested directly from infected fish was not superior antigenically to vaccines produced from cultured organisms. Fish immunised with this "wild" antigen died gradually when they were cohabited with infected fish two and a half month after immunisation. The most probable explanation for lack of immunity when cultured *Paramoeba* was used, was thought to be the absence of virulence factors in the cultured organisms. It is difficult to explain why a crude vaccine containing antigens from virulent organisms did not work. However, it is possible that the antigenic determinants in the crude antigen was covered by gill tissue components in this vaccine. Furthermore, it is shown that high serum antibody level against *F. branchiophilum* do not relate to the degree of protection against the experimental disease (Lumsden *et al.*, 1994) and the same situation could well apply with *Paramoeba* sp. Further research is needed to elucidate this.

Regarding the lack of protectiveness of mammalian sera in combating paramoebiasis, it should not be forgotten that in cohabitation, 100% morbidity was not expected. With such a high mortality level it is difficult to assess the protection efficacy. Despite the above limitations, fish ELISA results monitoring the antibody levels of fish exposed to *Paramoeba* antigens were promising in detecting fish antibody due to the disease for diagnostic purposes.

No significant difference was observed between the antibody levels of naturally and experimentally infected fish with paramoebiasis. Thus, long exposure of fish does not seem to stimulate an enhanced immune response. Therefore, the results obtained from *Paramoeba* trial investigation have considerable potential value in relation to future immunisation trials, protection experiments and immunological assays. Further work is required to elucidate immunological aspects of AGD, possibly determination of antigenic components of *Paramoeba* sp., local antibody production in gill tissues and secretions (by immersion vaccination) and genetic engineering may help to improve vaccines and/ or by manipulating the *Paramoeba* to deplete its virulence in future to overcome such a problematic and important fish disease.

Generally, hyperimmune sera act as antimicrobials, but, unlike antibiotics that usually last 10 days or less, hyperimmune sera provide protection for 22 to 359 days against furunculosis and other diseases. As they are immunoglobulins, there is no danger of resistance development, residue problems, or the need for withdrawal times (Turgeon and Elazhary, 1992).

Production of engineered antibodies for therapy is under investigation (Adair, 1992). The biotechnology industry is beginning to furnish these products. The production of antibodies, in particular monoclonal antibodies may play a vital role in the future of therapeutic against infectious and non-infectious diseases.

Regarding the cost of preparation of hyperimmune sera in mammals or on tissue cultures against fish pathogens and comparing the cost of bacterins for vaccination of fish against infectious diseases of fish, it can be concluded that passive immunisation

is cost-effective. Since each fish can be immunised with a small amount of immune serum (0.1 ml), therefore thousands of fish can be immunised by 1 litre of serum prepared in a mammal.

It is obvious from this study that passive immunisation has a significant potential when given to the fish at strategic points where there is not enough time for active vaccination. Thus, there should be a future for the use of mammalian hyperimmune sera or genetic engineered antibodies against fish diseases, in particular, those for which an optimum immunity by active immunisation is not available.

Summary of the Thesis

Passive immunisation of fish was carried out to determine whether anti-*Vibrio anguillarum* antibodies (AVA), anti-*Streptococcus* sp. (anti streptococcal) antibodies (ASA), and anti-*Paramoeba* sp. antibodies (APA) produced in sheep, rabbits and fish were persistent and biologically active in fish. Results of passive immunisation were compared with results of active immunisation in which fish were immunised by immersion and injection with formalin-killed cells. Assessments of passive and active immunisation were carried out concurrently for up to three months in order to demonstrate their relative efficacies and, especially, to evaluate the practical potential of passive vaccination.

Experiments were conducted to determine the rate of clearance of sheep, rabbit and fish AVA levels, from the sera of rainbow trout (*Oncorhynchus mykiss*, Walbaum) at various periods after an intraperitoneal (i.p.) injection. A sensitive enzyme-linked immunosorbent assay (ELISA) was employed to detect longevity of the transferred antibodies. Results showed that the peak antibody level of sheep AVA appeared at 2 hours (h) and rabbit and fish AVA at 6 h after immunisation. Both rabbit and sheep antibodies persisted for up to 70 days in fish sera after an i.p. injection, whereas fish antibodies persisted no more than 35 days.

When the level of antibodies (sheep and rabbit AVA) from passive immunisation were compared with antibodies produced endogenously after active immunisation, it was found that the ELISA optical densities at 3-8 weeks after immunisation were very similar in fish immunised by either means. However, the actively-immunised fish maintained their antibody level while the passively-immunised fish progressively lost theirs.

Passive and active immunisation of Atlantic salmon (*Salmo salar*) against *V. anguillarum* were carried out and assessed 4 and 6 weeks after immunisation. Both passively and actively immunised Atlantic salmon showed antibody levels similar to those of rainbow trout.

Studies were also undertaken to monitor the immune responses to the heterologous (sheep and rabbit) sera. Using specific ELISA's against these sera, anti-sheep and anti-rabbit serum antibodies were detected in fish sera with the greater response being to the rabbit serum. However, even when multiple doses of heterologous sera were used there was no clinical evidence of anaphylaxis.

Relative levels of protective immunity were determined by i.p. challenge of fish with virulent *V. anguillarum*. Regarding the protective value of anti-*Vibrio* antibodies, sheep AVA significantly protected rainbow trout one month post-immunisation, moderately at 2 months and marginally at three months. The relative percent survival (RPS) of challenged fish after an i.p. injection of sheep, rabbit or fish AVA was 93.3, 86.6, 40.0 at one month (75% mortality in non-immunised control group = MNICG); 25, 18.7, 12.5 at two months (80% MNICG) and 13.3, 4.1, 9.0 at three months (73% MNICG) post-immunisation respectively. Fish actively immunised by immersion

showed RPS of 86.6, 93.0, and 81.8 after one, two and three months post-immunisation in the same trials respectively.

In order to obtain equivalent protection to undiluted fish serum, rabbit and sheep sera had to be diluted 1: 8 and 1: 50 respectively.

Protection conferred by immune sera was shown to be due to the specific antibodies alone. Sheep and rabbit AVA sera were purified by affinity chromatography using protein G and protein A respectively and were demonstrated to have equivalent protective effect to the whole antisera in rainbow trout. Also heat treatment of sera to inactivate complement had no effect on the potency of either immune or non-immune sera.

Oral delivery of AVA to rainbow trout was attempted using protein G purified antiserum prepared in sheep and purified antibodies conjugated to the carriers Quil A, TraT (immuno-stimulatory carriers, an internal membrane of *E. coli*) and LTB (the GM-1-binding subunit of *E. coli* heat-labile toxin). No AVA were detected at 24 h, 7 days, 30 days, and 60 days after dosing. However, fish dosed with AVA conjugated to LTB had a relative percentage survival of 37.5% at 15 days after dosing and 27% after one month. As LTB by itself had no protection effect, it was presumed that protection was associated with specific antibodies bound to receptors at key sites such as the spleen, liver and kidney. Fish response to sheep AVA and also to Quil A, TraT and LTB as immunostimulants was determined using specific ELISAs. No significant fish response to either the sheep antisera or the immunostimulants was observed .

In the second approach of the research the clearance rates of sheep, rabbit and fish anti-streptococcal antibodies from rainbow trout were monitored. A sensitive ELISA was developed to detect longevity of transferred antibodies. Results showed that sheep antibodies in rainbow trout sera were more persistent than rabbit antibodies. The peak antibody level of sheep and rabbit ASA in the circulation was at 24 h after passive immunisation. Sheep antibodies persisted well up to 60 days, but were present at a lower level at 75 days. Rabbit antibodies persisted up to one month after an i.p. injection while fish antibodies could not be detected at 6 h post-immunisation. A similar trend for anti-*Streptococcus* sp. antibody was observed when it was compared with anti-*Vibrio* antibody. Both sheep and rabbit ASA showed marked persistence.

Rainbow trout immunised against *Streptococcus* sp. with formalin-killed cells by an i.p. injection had a detectable antibody level at 6 weeks after immunisation, but the response in immersion-vaccinated fish was not detectable.

Anti-streptococcal antibodies prepared in sheep and rabbits significantly protected fish against streptococcosis at one month after immunisation, moderately (in the case of sheep) at two months and marginally (also in the case of sheep) after three months. The relative percent survival of challenged fish after an i.p. injection of sheep, rabbit and fish ASA was 88.8, 50.0, 0.0 after one month (75% mortality in non-Immunised control group = MNICG, 32-42 cfu of *Streptococcus* sp.); 33.3, 6.8, 6.8 after two months (85% MNICG, 55-65 cfu of *Streptococcus* sp.) and 13.3, 0.0, 6.6

after three months (62.5% MNICG, 4-7 cfu of *Streptococcus* sp.) post-immunisation respectively.

Results showed that fish immunised by i.p. injection using formalin-killed *Streptococcus* sp. cells had a high level of resistance. However, those immersed in the bacterin had very low or no protection. The relative percent survival for injected and immersed fish was 88.8 and 11.1 after one month, 38.1 and 4.7 after two months 36.0 and 0.0 after three months post-immunisation.

Thus, passive immunisation shows potential for protection against such a disease as it gives similar results to injection vaccination and would be expected to provide immediate protection.

In the third approach, Atlantic salmon (*Salmo salar*) immunised with an immune sheep anti-*Paramoeba* antibodies (APA) were bled and tested for the rate of clearance of these antibodies from fish sera. Using a specific ELISA, sheep APA showed a high level which cleared over a 2 months period in the fish sera .

Groups of fish were vaccinated i.p. with several *Paramoeba* antigens; killed cultured P.A with and without Freund's complete adjuvant (FCA). Fish were bled and tested for fish APA using specific ELISA. Results showed significant elevation of APA in fish sera following vaccination of fish with formalin killed and sonicated *Paramoeba*. The marked humoral response following vaccination indicated a significant effect of adjuvants in stimulating the fish's immune response.

Naturally and experimentally infected fish were tested by ELISA. Naturally infected fish with AGD showed low APA which were not significantly different from experimentally infected fish. However, the fish antibody level in both groups were higher than that of control fish.

A *Paramoeba* transmission method was established to assess the protection afforded by different methods of immunisation. Cohabitation of infected Atlantic salmon with previously-unexposed (naive) Atlantic salmon was successful.

Seven to ten days after introducing infected fish (5% of fish population in 4000L tanks), classic AGD patches were observed, however the organism was hardly seen. With the progress of the disease *Paramoeba* sp. could be seen in typical form. However, protectiveness of sheep antibodies conferred into the fish and those produced by fish could not be demonstrated due to a disease outbreak (morbidity of 100%) in the experimental challenge trial. No unequivocal protection was demonstrated in any of the vaccinated fish.

It is shown in this study that passive immunisation has significant potential when given at strategic times especially when there is not enough time for active vaccination. Thus, there should be a future for the use of mammalian hyperimmune sera against fish diseases, in particular, those for which practical immunity by active immunisation is not available.

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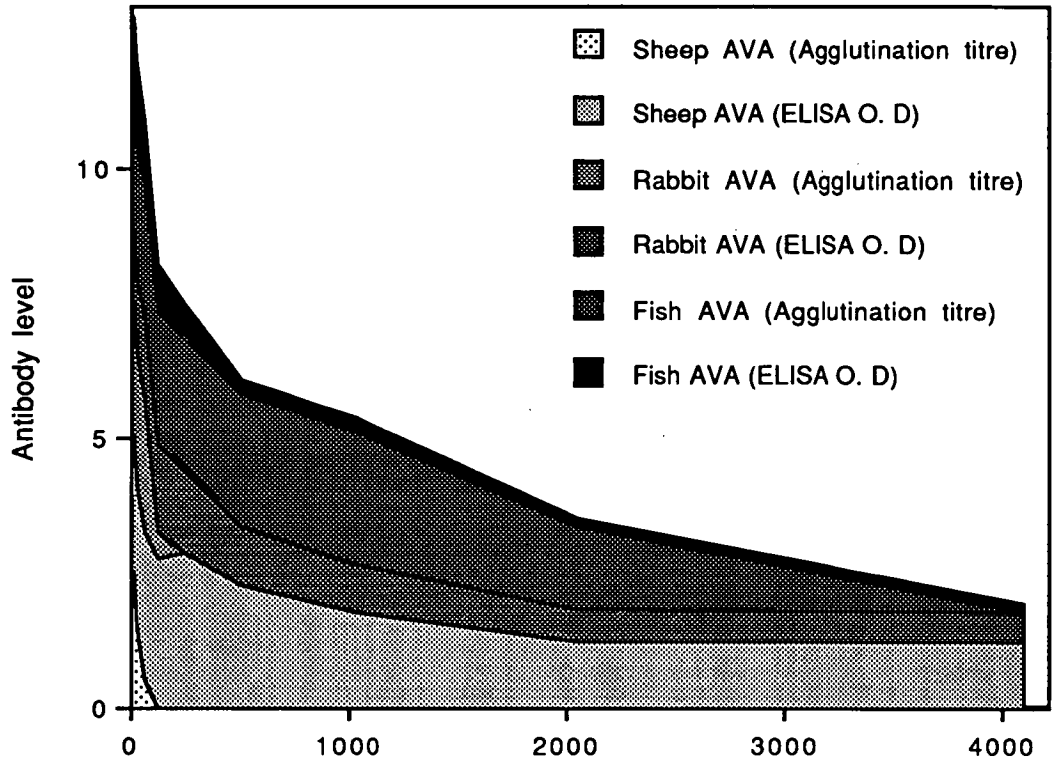
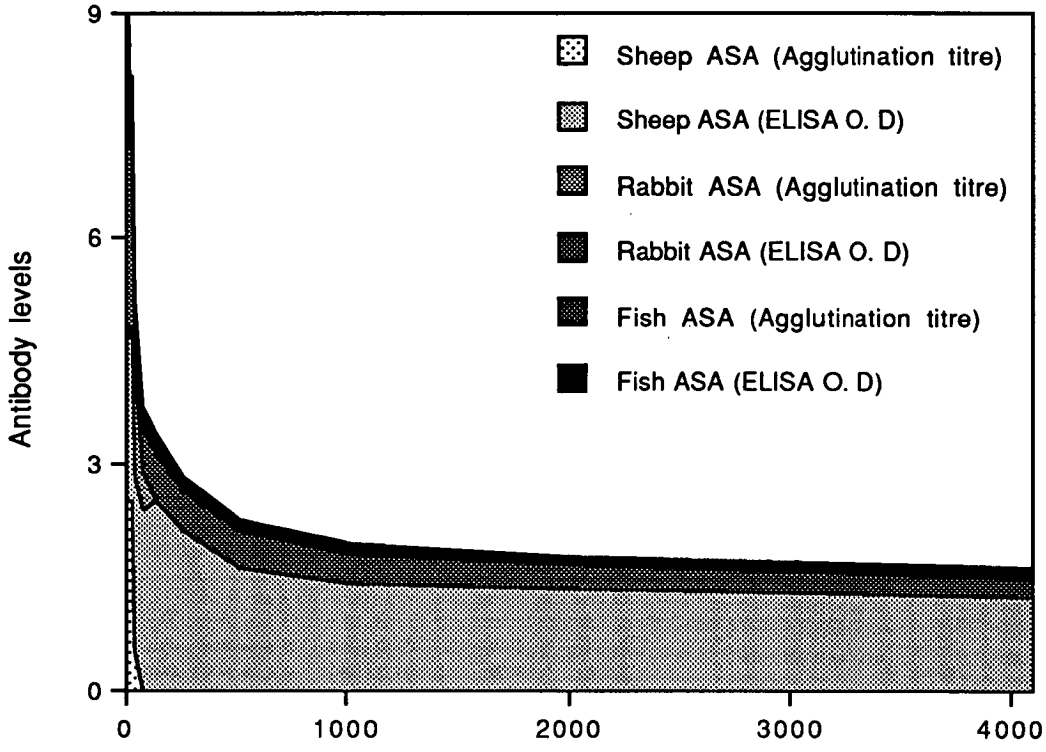
Appendix I: Abbreviations

AGD	Amoebic gill disease
APA	Anti-*Paramoeba* sp. antibodies
ASA	Anti-*Streptococcus* sp. (streptococcal) antibodies
AVA	Anti-*Vibrio anguillarum* antibodies
BA	Blood agar
BHI	Brain heart infusion agar
cfu	Colony forming unit
DWT	Distilled water+ 0.05% Tween 20
ELISA	Enzyme-linked immunosorbent assay
EMAI	Elizabeth Macarthur Agricultural Institute
FITC	Fluorescein isothiocyanate conjugated antibody
h	Hour
HRP	Horse radish peroxidase
IFAT	Immunofluorescent antibody test
i.p.	Intraperitoneal injection
L	litre
LPS	Lipopolysaccharide
LTB	The GM-1-binding subunit of *E. coli* heat-labile toxin
m	Month (s)
min	Minute
mm	Millimetre
O.D	Optical density
P.A	*Paramoeba* sp.
P-N	Positive-negative
PBS	Phosphate-buffered saline
PBSTG	Phosphate-buffered saline+ 0.05% Tween 20+ 0.1% Gelatin
p.i.	Post-immunisation
r.p.m	Revolution per minute
RT	Room temperature
SALTAS	Salmon Enterprises of Tasmania
S.D.	Standard deviation
SDS Page	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
Sec	Second
S.E.	Standard error
TraT	An internal membrane of *E. coli*
w	Week
WCB	Whole cell bacteria

Appendix II: Agglutination titre and ELISA antibody levels for AVA and ASA (shown by area)



Antibody levels vs dilution of sheep, rabbit and fish AVA measured by agglutination (Agglutination of+++ has calculated as 2.5, ++ as 1.5, + as 0.5 and - as 0) and ELISA O.D has been shown by area



Antibody levels vs dilution of sheep, rabbit and fish ASA measured by agglutination (Agglutination of+++ has calculated as 2.5, ++ as 1.5, + as 0.5 and - as 0) and ELISA O.D has shown by area

Appendix III: ELISA reagents

Sera:

Whole, ammonium sulphated precipitated or affinity purified sera

Conjugates:

1- KPL Rabbit anti-sheep Ig-HRP, peroxidase conjugated and affinity purified antibody to sheep IgG (H+L).

2- DAKO Peroxidase-Conjugated Rabbit immunoglobulins to Mouse Immunoglobulins and affinity purified.

3- DAKO Peroxidase-Conjugated Swine immunoglobulins to rabbit Immunoglobulins and affinity purified.

Substrate

ABTS (Sigma) 2,2'-Azino-bis (3-Ethyl benzen thiazoline-6-sulfonic acid) Diammonium salt (0.55 g) + citric phosphate buffer (1L).

Citrate phosphate buffer: citric acid 21.0 g, Na2HPO4 14.0 g and MQ water to 800 ml ; adjust pH to 4.2; make up to 1L.

Substrate stop solution:

Citric acid 10.5g, MQW to 500 ml; add 50 mg sodium azide or 1 ml of 5% solution.

Other solutions:

10x TRIS-saline

(250 mM Tris, 1.5 M NaCl)
15.14 g Tris
43.83 g NaCl
500 ml Milli-Q water
pH 7.4

TS Methanol

10 x Tris saline	100 ml
Milli-Q water	800 ml
Methanol	100 ml

TSGM

10 x Tris- saline pH 7.4	50 ml
Glycerol 250 ml	
Milli Q water to make	500 ml
Autoclave	
Add 1 ml 10% Merthiolate	
Store in dark place	

Borate coating buffer

Boric acid	6.18 g
Disodium tetraborate	
Na2B4O7.10H2O	9.54 g
NaCl	4.38 g
MQ water to	1L
Autoclave	

Phosphate buffered saline

NaCl	8.0 g
KCl	0.2 g
Na2HPO4	1.15g
KH2PO4	0.2 g

MQ water to 900 ml; pH to 8.0 with HCl or NaOH; make up to 1L

Autoclave

PBST

Tween 20	0.5 ml
PBS pH 8.0	1000 ml

PBSTG

PBST	1000 ml
Gelatin	1 g

Appendix IV: ELISA procedures (summarised)

Procedures for sheep and rabbit ELISA:

1- Coating the ELISA plate:

Vibrio antigen (WCB) diluted 1:100 in TS Methanol; 100 µl well^{-1}

Spinning at 2500 rpm, for 10 min. Flicking antigen off.

2- Blocking 1% gelatin in PBST 100 µl well^{-1}; require 10 ml per plate

Incubating for 30 min. room temperature (RT) and washing.

3-Adding sheep/rabbit serum dilution of 1: 200 (neat and ammonium sulphate purified prebleed and immune serum diluted in two fold basis with PBSTG in duplicate wells.

Including control +ve highly positive, medium positive, positive and negative (diluted in negative serum). 100 µl well^{-1}.

Incubating 90 min. in RT and washing.

4- Adding conjugate, Kirkegard Perry Laboratories (KPL) Rabbit anti-sheep Ig-HRP (polyclonal rabbit anti-sheep)in dilution of 1:2000 (sheep ELISA), Dako swine anti-rabbit in dilution of 1:750 (rabbit ELISA) and incubating 90 min.RT and washing.

5- Adding ABTS substrate (1 mM 2,2'- azino-di (3-ethyl-benzthiazolin-6-sulphate)) and 0.03% H2O2 dissolved in citrate buffer (22ml+10 µl H2O2), 100 µl well^{-1} to detect bound conjugated antibodies. Incubating 20 min. RT on shaker.

6-Stopping the reaction with ABTS stop solution, 50 µl well^{-1} and reading the colour spectrophotometrically at 405 nm by computer programmed microplate reader.

Procedure of ELISA for rainbow trout

In this assay all the diluents for serum, monoclonal antibody (MAb) and conjugate should be adjusted at pH 8.0.

1- Coating the ELISA plate:

Vibrio antigen (WCB) diluted 1:100 in TS Methanol 50 µl well^{-1}

Spinning at 2500 rpm, for 10 min. Flicking antigen off.

2- Blocking 1% gelatin in PBST 100 µl well^{-1}; require 10ml per plate

Incubating for 30 min. room temperature (RT) and washing.

3-Adding fish serum (neat prebleed and immune serum in 1:100 dilution with PBSTG in duplicate wells.

Including control +ve highly positive, medium positive, positive and negative (diluted in negative serum). 50 µl $well^{-1}$.

Incubating 90 min. in RT and washing.

4- Adding mouse monoclonal antibody to trout IgM ; 50 µl $well^{-1}$.

Incubating 90 min. in RT and washing.

5- Adding conjugate, Dako Rabbit anti-mouse-HRP; 50 µl $well^{-1}$

Incubating 90 min.RT and washing.

5- Adding ABTS substrate (22ml+10µl H_2O_2), 50 µl $well^{-1}$. Incubating 20 min. RT on shaker.

6-Stopping the reaction with ABTS stop solution, 25 µl $well^{-1}$ and reading the colour at 405 nm by computer programmed microplate reader.

Appendix V: Buffers and solutions for affinity chromatography (stored at 4°C)

Starting buffer:

Tris base	3.02 g
NaCl	8.76 g
Distilled water to	900 ml
Na azide 5%	2.0 ml

pH adjusted to 7.2
Making up to 1000 ml

Elution buffer:

Glycine	7.51 g
NaCl	8.76 g
Distilled water to	900 ml
Na azide 5%	2.0 ml

pH adjusted to 3 with concentrated HCl
Making up to 1000 ml

2M Tris buffer

Tris base	24.22 g
Distilled water to	100 ml

Tris regeneration buffer

Tris base	12.11 g
NaCl	29.22 g
Distilled water to	900 ml
Na azide 5%	2.0 ml

pH adjusted to 8.5 with NaOH
Making up to 1000 ml

Acetate regeneration buffer

Na acetate	8.20 g
NaCl	29.22g
Distilled water to	900 ml
Na azide 5%	2.0 ml

pH adjusted to 4.5 with HCl
Making up to 1000 ml

Appendix VI: ELISA O.D results: maximum, minimum and cut-off values of fish to *V. anguillarum* and *Streptococcus* sp. cells

Fish antibody responses after i.p. injection of 1 mg (dry weight) formalin killed *Vibrio* cells

Fish status positive/negative	ELISA O.D mean	S.D.	min	max	cut-off
Control fish					
Wk-3	0.158	0.021	0.067	0.172	_
Wk-5	0.164	0.030	0.075	0.235	_
Wk-8	0.104	0.024	0.071	0.143	_
Wk-10	0.093	0.032	0.067	0.108	_
Immunised fish					
Wk-3	0.439	0.196	0.221	0.843	0.199
Wk-5	0.438	0.253	0.245	0.890	0.224
Wk-8	0.409	0.228	0.172	0.772	0.152
Wk-10	_	_	_	_	_

Fish antibody responses after i.p. injection of 1 mg (dry weight) formalin killed *Streptococcus* sp. cells in 50% v v $^{-1}$ FCA

Fish status	ELISA O.D mean	S.D.	min	max	positive/negative cut-off
Control fish					
wk-4	0.165	0.104	0.067	0.195	_
wk-6	0.156	0.087	0.163	0.186	_
Immunised fish					
wk-4	0.341	0.124	0.222	0.446	0.209
wk-6	0.401	0.067	0.225	0.445	0.200

Appendix VII: Anti-microbial compounds used in Aquaculture for treatment of vibriosis and their method

Antimicrobial compound	Methods of administration and dai ly dosage			comments	Persistence in fish tissues	Reference
	by food	by bath	by injection			
Chloramphenicol	50-70 mg/kg b.w/ day for 5-10 days	10-50 mg/L of water as a bath				Austin &Austin(1993)
				Public health concerns		Munday (1993)
Flumequine	6 mg/kg b.w/day for 6 days					Austin &Austin(1993)
	20 mg/kg b.w for 10 days in seawater		9 mg/kg b.w for sharks	256 h half life after single injection	60-80 days	Munday (1993)
Furance	2-4 mg/kg b.w /day for 3-5 days	0.5-1 mg/L of water for 5-10 min, as a bath				Austin &Austin(1993)
Furazolidone	25-75 mg/kg b.w/day for up to 20 days					Austin &Austin(1993)
				Deteriorates rapidly in wet (raw fish) diet		Munday (1993)
Kanamycin	50 mg/kg b.w for 7 days					Austin &Austin(1993)

b.w= body weight

Antimicrobials against vibriosis (continued)

Antimicrobial compound	Methods of administration and dai ly dosage			comments	Persistence in fish tissues	Reference
	by food	by bath	by injection			
Erythromycin	75-125 mg/kg of b.w for 10-21 days					Munday (1993)
Oxytetracycline	50-70 mg/kg b.w/day for 10 days					Austin &Austin(1993)
				Chelates with divalent cations May interfere with immune response and osmoregulation in smolts	22 (70 days for skin)	Munday (1993)
Sodium nifursty-renate	50 mg/kg b.w/day for 3-5 days					Austin &Austin(1993)
Tetracycline	75-100 mg/kg b.w/day for 10-14 days					Austin &Austin(1993)
Nifurprazine hydrochloride	10 mg/kg of food, fed for 3-6 days	0.01-0.1 mg/L of water, as a bath for an undefined period				Austin &Austin(1993)

b.w= body weight

Antimicrobials against vibriosis (continued)

Antimicrobial compound	Methods of administration and dai ly dosage			comments	Persistence in fish tissues	Reference
	by food	by bath	by injection			
Nitrofurantoin		50 mg/L of water, as a bath for 1 h				Austin&Austin(1993)
Oxolinic acid	20-35 mg/kg b.w/day for 10 days in seawater 10-15 mg/kg b.w/day for 10 days in freshwater	1ppm for 24 h or 50 ppm 1 h			12-38 days	Munday (1993)
Oxytetracycline hydrochloride			60 mg/kg b.w as a single i.p. injection	chelates with divalent cations May interfere with immune responses and osmoreglulation in smolts	22-70 days	Munday (1993)
Sulphamerazine	100-200 mg/kg b.w for 10-20 days					Austin &Austin(1993)
Sulphamethazine	"					
Sulphisoxazol	"					
Tribrissen (potentiated sulphonamide)	50 mg/kg b.w/day for 10 days		20 ppm for 5-7 days			Munday (1993)

b.w= body weight

Appendix- VIII

Immunostimulants, adjuvants and vaccine carriers (Adapted from Anderson, 1992)

Reservoirs and depots:
- Freund's Complete Adjuvant (FCA)
- Freund's Incomplete Adjuvant (ICA)
- Liposomes
- Mineral oil
- Lanolin
- Paraffin oil
- Dextran sulphate
- Ethylene-vinyl acetate

Carriers and vehicles:
- Bentonite
- Kaolin
- Latex beads
- Sheep Red Blood Cells (SRBC)
- Dimethylsulfoxide (DMSO)

Inflammatory agents:
- Silica particles
- Carbon particles

T cell stimulator:
- *Mycobacterium* sp.
- Muramyl dipeptide (MDP)
- Glucans (yeast extracts)
- Metallic salts
  - Alum (potassium aluminum sulphate)
  - Aluminum hydroxide gel
- Levamisole
- Bacille Calmette Guerin
- *Corynebacterium parvum*

Polynucleotides:
- Poly A-U
- Poly I-C
- Isoprinosine
- Fluro-quindone

B cell stimulators:
- Lipopolysaccharides

Cell membrance modifiers
- Detergents
- Sodium dodecyl sulphate (SDS)
- Quaternary ammonium compounds (QAC)
- Saponins (plant extracts)
  - Quil A

Animal extracts:
- *Ecteinascidia turbinata* extract (Ete)
- *Haliotis discus* extract (HDe)
- Fish extract (ISK)

Mitogens (lectins, plant extract)
- Pokeweed mitogen (PWM)
- Phytohemagglutinin (PHA)
- Concanavalin A (Con A)

Nutritional factors:
- Vitamin C
- Vitamin E

Cytokines:
- Interleukins:
  - Leukotriene B_4
  - Interferon

Heavy metals:
- Cadmium
- Germanium

List of published/ submitted papers

Akhlaghi M. 1993. Passive immunisation of fish: potential for aquaculture. Austasia Aquaculture. 7: 47-48.

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