Final Report

AMOEBIC GILL DISEASE (AGD) VACCINE DEVELOPMENT
PHASE II – MOLECULAR BASIS OF HOST PARASITE
INTERACTIONS IN AMOEBIC GILL DISEASE

Mathew Cook, Nicholas Elliott, Giles Campbell, Jawahar Patil, Bronwyn Holmes, Vivian Lim and Chris Prideaux

June 2008

Aquafin CRC Project 3.4.4(2)
(FRDC Project No. 2004/217)

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Australian Government
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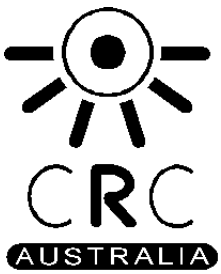

2004/217	**Amoebic Gill Disease (AGD) Vaccine Development Phase II– Molecular Basis of Host Parasite Interactions in Amoebic Gill Disease**

1. NON-TECHNICAL SUMMARY

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OBJECTIVES:

1. To identify potential protective antigens from *N. pemaquidensis* using a combined DNA/protein approach.
2. To demonstrate protection of Atlantic salmon against clinical amoebic gill disease via cDNA and/or recombinant protein vaccination.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE
The most significant outcome of this project is the commencement in July 2007 of sea trials for the experimental DNA vaccine made up of six antigens shown to provide a relative increase in protection of approximately 40 percent in laboratory based amoebic gill disease (AGD) trials. The six clone vaccine is protected by patents as follows: filed on 1st October 2007, in Australia as Application No. 2007221759, in USA as Application No. 11/906,440, and in Canada – Application No. 2605130.

- Expression library immunisation (ELI) of Atlantic salmon followed by acute AGD challenge identified one sub-library that provided a significant increase in protection.
- Bio-informatic analysis and further sub-library challenges identified a group of six DNA antigens that consistently provided approximately 40 percent increase in protection against morbidity in acute AGD challenge experiments.
- A homologous promoter (Atlantic salmon β-actin) and a heterologous promoter (CMV) driving foreign gene expression in Atlantic salmon muscle cells were able to achieve similar levels of protection with a six clone DNA vaccine.

- There was evidence that the six clone vaccine is able to significantly reduce both the crude gill score (visual assessment of percentage of gill surface affected by the parasite) and percentage of affected filaments in Atlantic salmon challenged with acute AGD infection.
- Incorporation of the Atlantic salmon Interlukin-1β gene as an adjuvant did not increase the level of protection provided by the six clone vaccine.
- All six DNA antigens are expressed as mRNA when injected either singularly or as a mix into salmon muscle tissue.
- Sera pooled from fish injected with the mix of the six DNA antigens are able to recognise a band corresponding to one of the clones (SC10) in western blot.
- Individual fish vary in their ability to produce antibodies to the SC10 antigen as measured by ELISA.
- All six DNA antigens were able to be expressed as HIS-tagged recombinant fusion proteins *in vitro* and could be successfully purified.
- A single attempt at vaccination with recombinant proteins representative of the six DNA antigens did not significantly increase the protection compared to vaccination with the delivery adjuvant alone.
- Further research is required on;
    - Assessing the crude vaccine under a chronic infection challenge in the sea under commercial growing conditions.
    - Refinement of the existing six clone vaccine and additional antigens that may increase efficacy.
    - Optimisation of the delivery vector.
    - Potential protection when using protein vaccination.
    - The nature of the response to the six antigens (humoral *versus* cellular immunity).
    - The optimum dose required.
    - Timing of vaccination prior to seawater transfer.

KEYWORDS:

Atlantic salmon, *Neoparamoeba*, DNA vaccine, recombinant protein, response

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Melanie Leef, School of Aquaculture, University of Tasmania

Richard Morrison, School of Aquaculture, University of Tasmania

Barbara Nowak, School of Aquaculture, University of Tasmania

For co-supervision of Masters student Vivian Lim (Chapter 8):

Richard Morrison, School of Aquaculture, University of Tasmania

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Harry King, SALTAS

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3. BACKGROUND

Amoebic gill disease (AGD) is considered to be the most significant health problem for farmed Atlantic salmon in Tasmania, costing the industry an estimated $15-20 million pa. It is caused by the presence of *Neoparamoeba* spp[1]. on the gills and if untreated can lead to death. Although some control of the disease is achieved by freshwater bathing, this procedure is not considered a viable, long-term solution. It is not fully efficacious – more than one treatment is usually required; it is very labour-intensive; it requires large volumes of freshwater, and is stressful to the fish, which further impacts on their health and growth. Furthermore, recent experience indicates an increase in the required frequency for freshwater bathing in an attempt to control the disease.

Vaccine development is part of a multifaceted approach to develop short-, medium- and long-term solutions to the control of AGD; development of a sub-unit vaccine being the long-term approach of the overall research strategy. The essential objective is to develop a set of strategies and tools to provide the salmon industry with a substantial reduction to the economic impact of AGD in an economically and environmentally sustainable way.

Vaccination is one of the most effective methods for controlling infectious diseases. Vaccines are commercially available for several bacterial pathogens, and considerable research has been conducted on vaccines for both bacterial and viral pathogens of fish. In contrast, relatively little has been done on vaccines against parasitic diseases. Recently significant advances have occurred in the development of vaccines for parasites in humans and traditional domestic animals, using either recombinant peptides, DNA

[1] A recent publication by Young et al (2007) identifies the possible causative agent as *Neoparamoeba perurans*. However, at time of submission Koch's postulates have not been fulfilled with regard to *N. perurans*. Furthermore, within this project no efforts were made to identify the amoebae used to induce AGD in the challenges, or to ensure a single species was present in challenge material. Therefore, within this report we have referred to the isolates used and the causative agent as *Neoparamoeba* spp.

vaccines or synthetic peptides. For example vaccines have been shown to have promise for protection against malaria (Sedegah et al., 1994), *Trypanosoma cruzi* infections (Costa et al., 1999) and cysticercosis (Manoutcharian et al., 1998). DNA vaccines have shown remarkable protection against IHN virus in salmonid fish (Anderson et al., 1996), but have yet to be utilised for fish parasites. Experimental recombinant vaccines were developed against the ciliate, *Ichthyophtirius multifiliis*, by identification of a surface immobilization antigen which protects the fish (He et al., 1997).

Experiments conducted prior to the establishment of the Aquafin CRC indicate that immunisation with crude, whole parasite preparations does not result in significant levels of protection from infection, despite the production of antibodies in the serum of treated fish (Zilberg and Munday 2001). Studies on parasitic diseases of other commercial species of animals have yielded similar results with crude antigen preparations; however, the use of subunit or single antigen preparations has resulted in the development of protective immunity in a number of well documented cases (Smith et al., 1994). Furthermore, in mammals the immune response to *Encephalitozoon cuniculi* has been described as CD8+/Tcell dependent (Didier and Bessinger 1999), with recovery and protection from re-infection not clearly characterized. A similar situation exists in AGD where recovery from infection has not been clearly demonstrated to protect from re-infection. The CD8+/Tcell dependent response observed in *Encephalitozoon cuniculi* does demonstrate the feasibility of developing an anti-parasitic subunit vaccine, even under these circumstances, although, to our knowledge, no commercial recombinant vaccine directed against protozoan diseases for humans, terrestrial animals or fish has been produced. Thus this project was viewed as high-risk since its conception and the development of such a vaccine would be ground breaking.

4. NEED

Health is one of the major issues associated with intensive culture industries, including aquaculture. Unhealthy stock relates to unhealthy industry – higher production costs, reduced viability, poor market and public image. Outbreaks of infectious salmon anaemia

(ISA) in Scotland resulted in some salmon companies going into receivership. The use of medication increases production costs and negatively impacts on the market.

In Australia, salmon aquaculture is relatively disease free, which provides a possible marketing advantage. However, Amoebic Gill Disease costs are ca 10% of the gross value of production; a significant production cost for farming Atlantic salmon in Tasmania. The disease outbreaks seem to intensify as the industry develops and now are prevalent not only during summer months but also in winter. Outputs of the CRC Health Program such as improved treatment of AGD infected fish, AGD risk forecasting ability and vaccine production against AGD will increase the profitability and competitiveness of the Australian salmon industry.

AGD is severely limiting further expansion of the industry because it ties up resources that could be directed elsewhere, and it limits farming sites due to reliance on freshwater for bathing. Current treatment of AGD while environmentally friendly is not viable in the long-term and alternatives are required. Other treatments may be more reliant on antibiotics or chemicals, a risk to the image of the industry. Although vaccine development can be seen as a high-risk research, the potential long term rewards justify the risk.

5. OBJECTIVES

1. To identify potential protective antigens from *N. pemaquidensis* using a combined DNA/protein approach.
2. To demonstrate protection of Atlantic salmon against clinical amoebic gill disease via cDNA and/or recombinant protein vaccination.

6. Expression Library Immunisation against amoebic gill disease (AGD)

6.1 Introduction

Expression library immunisation (ELI) is a powerful tool that can be utilised to systematically screen the genome of a given pathogen to identify potential vaccine candidates (Talaat and Stemke-Hale, 2005). The ELI approach enables either genomic DNA (gDNA) or cDNA (complementary DNA, representative of the pathogens transcriptome or mRNA) to be cloned into a vector, or plasmid, under the control of a eukaryotic promoter. Theoretically this library is a true representation of all genes of the pathogen. Purified DNA from this library is then injected into animals, usually as sub-fractions, to induce a response against potential antigen/s. Injected animals are then challenged with the specific pathogen in order to determine which fractions of the library confer protection. Protective fractions can then be further refined until either a single antigen or a small pool of antigens are identified (Johnston and Barry, 1997). ELI offers the advantage of being a rapid screening protocol for many antigens while at the same time utilising protection as a measure, which ultimately is the aim of vaccination regimes (Talaat and Stemke-Hale, 2005). Indeed, ELI shows promise as a means of developing vaccines for diseases for which traditional vaccination methods have not been successful, in particular parasitic infections (Barry, *et al.* 1995).

Amoebic gill disease (AGD) caused by the amoeba, *Neoparamoeba* spp., continues to be the most important health issue affecting the culture of Atlantic salmon (*Salmo salar*) in Australia (Munday *et al.* 2001). Currently, AGD is treated by freshwater bathing. This treatment is repetitive, labour intensive, time-consuming, stressful to fish and costly. Furthermore, given the increased pressure on freshwater resources in Australia, this practice may not be sustainable in the long-term and alternative approaches, such as vaccines are required. There have been previous efforts to vaccinate against AGD. For instance, active immunization has been attempted using intraperitoneal (IP) injection or anal intubation of both live and sonicated wild-type or cultured *Neoparamoeba* either in

the presence or absence of adjuvants such as Montanide™ (Akhlaghi et al., 1996; Zilberg and Munday, 2001). Bath exposure to either avirulent cultured or infective wild type antigens has also been attempted, but failed to provide protection (Morrison and Nowak, 2005). Passive immunisation using antisera produced in sheep and rabbits has also been utilised in an attempt to immunize fish (Akhlaghi et al., 1996). There are contradictory reports on the ability of fish to acquire natural resistance following exposure. For instance, Findlay et al (1995) suggested that fish previously exposed to *Neoparamoeba* spp. were resistant to re-infection, as did Vincent et al (2007). However, Gross et al (2004) found that previously exposed fish were no more resistant to AGD than naïve fish. Therefore, despite the presence of anti-*Neoparamoeba* antibodies, vaccination studies have failed to demonstrate increased resistance or protection as measured by reduced gill scores (measure of percentage of gill area affected) or an increase in survivability following AGD challenge.

Neoparamoeba is a complex organism with a dense glycocalyx outer layer about 10 nm thick on its cell surface (Dyková et al. 1999). Possible reasons for the failure to demonstrate protection via vaccination include the pathogen hiding protective antigens from the host immune system, suggesting that immunodominant antigens, such as complex carbohydrates on the cell surface may mask or block the host antibody response, and the possibility of antigen shifting on the surface of *Neoparamoeba* spp.. It has also been proposed that the pathogen may mediate or suppress the host response (Gross et al., 2004). Other more technical reasons may be that avirulent cultures have been used or that the type of immune response generated (eg humoral) is inappropriate.

Given the lack of progress with previous AGD vaccine efforts and the inherent advantage of ELI for use with parasitic pathogens, ELI may offer promise as a method for discovering potential protective antigens for the development of a vaccine against AGD. In project 3.4.4 (FRDC project 2002/251) we developed a delivery vector and a full length cDNA library from a Wild Type infective gill isolate and the purpose of this work was to utilise this library in an ELI regime.

6.2 Methods

6.2.1 Plasmid Vector and cDNA Expression Library construction

The construction of the expression vector, pbS-lox and of the full length cDNA library were previously detailed in the Final Report for project 3.4.4 (FRDC project 2002/251).

6.2.2 Library sub-fractionation, culture and plasmid preparation

The wild-type cDNA library, contained in the vector, pbS-lox, was plated out onto 150 mm agar plates containing 50 µg/ml ampicillin and allowed to grow overnight at 37°C. Individual colonies were picked into 1.5 ml LB/Amp (50 µg/ml) culture media in 96-well deep well plates and allowed to grow overnight at 37°C; following this, 1 ml from each well of 3 plates (288 separate clones) were combined and fresh LB/Amp (50 µg/ml) media was added to a final volume of 500 ml and allowed to incubate on a shaker platform at 37°C and 150 rpm for a further 4 hours. Cultures were harvested while in mid log phase and subjected to an endo-free plasmid preparation (Endo-free Megaprep Kit, Qiagen, The Netherlands) as per the manufacturers instructions. Glycerol to a final concentration of 10% was added to the remaining 500 µl and the plates stored at -80°C for use as stock plates. A total of 1100 individual clones was picked and processed this way, resulting in the construction of three 288-clone and one 236-clone experimental ELI vaccine groups.

A control vaccine, consisting of only the pbS-lox vector backbone, was made by streaking out pbS-lox from glycerol stock on a pre-warmed LB/amp agar plate and growing overnight at 37°C. A single colony was picked into 5 ml of LB/Amp and allowed to grow overnight at 37°C, 500 µl was used to inoculate 500 ml of LB/Amp and the culture was allowed to grow for 4 hrs at 37°C. Cells were harvested while in logarithmic phase of growth and pbS-lox was purified using an endo-free Megaprep kit (Qiagen, The Netherlands) as per manufacturers instructions. In preparation for the vaccine trials each of the 4 library sub-fractions and the control vaccine, pbS-lox were diluted in sterile phosphate buffered saline (PBS) at a concentration of 10 µg DNA/50 µl.

6.2.3 AGD Challenge Trial 1

The AGD challenge trials were conducted by the School of Aquaculture, University of Tasmania, Launceston. Three hundred pre-smolt Atlantic salmon (*Salmo salar*) were acclimated to 16°C and 36 ppt seawater. Fish were marked by tattooing on the underbelly with alcian blue (0.5%) and separated into 6 groups (50 fish/group). Fish were then injected in the dorsal musculature with 50 µl of either phosphate buffered saline (PBS) (negative control), pbS-lox vector backbone (vector control, total 10 µg plasmid DNA) or one of the four library sub-fractions (total 10 µg DNA/fish). The fish were then further allocated equally across two tanks (25 fish/group/tank). Fish were given a booster injection at the same dosage, 14 days after the first injection and held for a further 14 days prior to challenge. Immediately prior to challenge, a total of 5 fish from each group were sacrificed and bled, and sera were collected and stored at -80°C for antibody analysis. Fish were challenged with a dose of 500 viable amoebae/L and monitored daily, with any moribund fish removed and their group recorded. Fish were fed at a rate of 1% body weight each day. The challenge was run until >60% overall morbidity was observed in the control group.

6.2.4 Sero-screening of Survivors in Trial 1

To facilitate further rationalisation of the best performing library fraction from trial 1, sero-screening of survivors was undertaken. Blood was collected by venipuncture, allowed to clot overnight, and centrifuged, and sera were isolated. Sera were stored at -80°C until used. All 288 clones from the best performing library fraction were grown overnight in 1 ml LB/Amp at 37°C. Following this, a 50 µl aliquot of overnight culture was used to inoculate 1 ml fresh LB/amp. Cultures were incubated at 37°C for 1 hr and then induced by addition of IPTG to a final concentration of 1 mM. Cultures were on-grown for 4 hr and cells harvested by centrifugation. Cells were lysed by addition of 100 µl BPER-II reagent (Pierce) as per the manufacturer's instructions. Proteins were immobilised on Nitrocellulose filters using a 96-well dot blot apparatus (BioRad) and applying 20 µl of the protein lysate under vacuum. The filters were then blocked using

TBS/Tween/BSA (TBS, 0.1% Tween, 3%BSA) for 30 min. Filters were washed with TBS/Tween, 3 x 5 min and incubated with a 1:50 dilution (in TBS/Tween) of fish sera pooled from all surviving individuals for 1 hr at room temperature. Filters were then washed in TBS/Tween, 3 x 5 min and then incubated with mouse-anti-salmon-Ig (Serotec) (1:250 dilution in TBS/Tween) for 1 hr at room temperature. Filters were finally washed 3 x 5 min and incubated with rabbit-anti-mouse-IgG-AP (1:1000 dilution in TBS/Tween) for a further 1 hr at room temperature. Following washing (TBS/Tween, 3 x 5 min, 100mM Tris-pH9 1 x 5 min) positive dot blots were visualised by addition of BCIP/NBT. Appropriate controls including incubation with naïve sera (sera from Atlantic salmon raised in freshwater) and incubation only with either secondary and/or tertiary antibodies only were also run. A blot was deemed positive if its colour was considered significantly darker than the equivalent spot blotted with normal sera.

6.2.5 AGD Challenge Trial 2

The second challenge trial was designed to further rationalize the best performing group from the Trial 1. In total there were six separate test groups consisting of two control groups (PBS and vector control), library fraction #2 (best performing fraction from Trial 1) and three sub-fractions of fraction #2. Two of the 'new' experimental groups were made by arbitrarily splitting in half fraction #2, thus providing two sub-fractions of 144 clones each. The third sub-fraction consisted of a mix of clones deemed positive in the sero-screeing procedure (see above). The procedure for vaccination and AGD challenge followed the same protocols as for Trial 1.

6.2.6 Statistical analysis

Relative percent survival (RPS) for each of the groups (compared to the negative control) was calculated using the following formula as per Amend (1981);

$$RPS = (1- (\%\text{vaccinated mortalities} / \%\text{control mortalities})) \times 100$$

Each vaccine group was also compared to both the negative control and vector only control using a log rank test (Bland and Altman, 2004).

6.2.7 Sequencing and Bio-informatic analysis

In parallel to the challenge trails, sequencing and bioinformatic rationalization of all the 288 clones in fraction #2 was undertaken to identify potential antigenic molecules. Briefly, each clone was picked individually into 2 ml LB/Amp and grown overnight at 37°C. Each clone was then subject to plasmid miniprep (Qiagen) as per the manufacturer's instructions. Approximately 100 ng of plasmid DNA was combined with 1 µl Big-Dye, 3.5 µl 5x sequencing buffer (ABI), 1 µl forward or reverse primer and distilled water in a total volume of 20 µl. The reaction was subjected to an initial denaturation step of 94°C/5 min followed by 25 cycles of 96°C/10 sec, 50°C/5 sec and 60°C/4 min. Products were then purified using CleanSEQ Dye-terminator removal kit (Agencourt Bioscience Corporation) as per the manufacturers instructions and applied to an ABI 3100 genetic analyzer.

Raw data from sequencing were transferred to ChromasPro V1.22 sequence analysis software. Vector sequences were trimmed automatically and the remaining sequence interrogated, PCR primer sequences and unreliable base calls were edited manually. The sequence data were then used to interrogate the NCBI (http://www.ncbi.nlm.nih.gov/) and protozoan pathogen specific databases *(Entamoeba histolytica, Plasmodium falciparum and Trypanosoma brucei),* using tblastx (translated nucleotide vs translated protein).

6.3 Results

6.3.1 Challenge Trial 1

During the vaccination and holding period there was a number of handling related mortalities across all groups in both tanks. Furthermore, there was a number of mortalities not related to AGD following challenge of the fish. These were most likely

due to a combination of water quality issues and a non-specific bacterial infection. All non-AGD related mortality data were excluded from subsequent analysis. AGD related morbidity began as early as five days post infection (DPI) with a logarithmic period of morbidity occurring from days 21 to 37. Morbidity rates were not significantly different between tanks, therefore data from both tanks were combined to generate the survival curves (Fig 6.1) and to undertake the log rank analysis. Only one of the sub-library vaccinated groups showed a significant increase in survivability ($p<0.05$). This group, fraction #2, had an average RPS of 36.5 and 31.5% compared to either the control or vector only groups respectively (Table 6.1). All other sub-libraries had varying RPS ranging from -14 to 28% (Table 6.1), none of which were significantly different from control fish using the analysis of the two tanks combined.

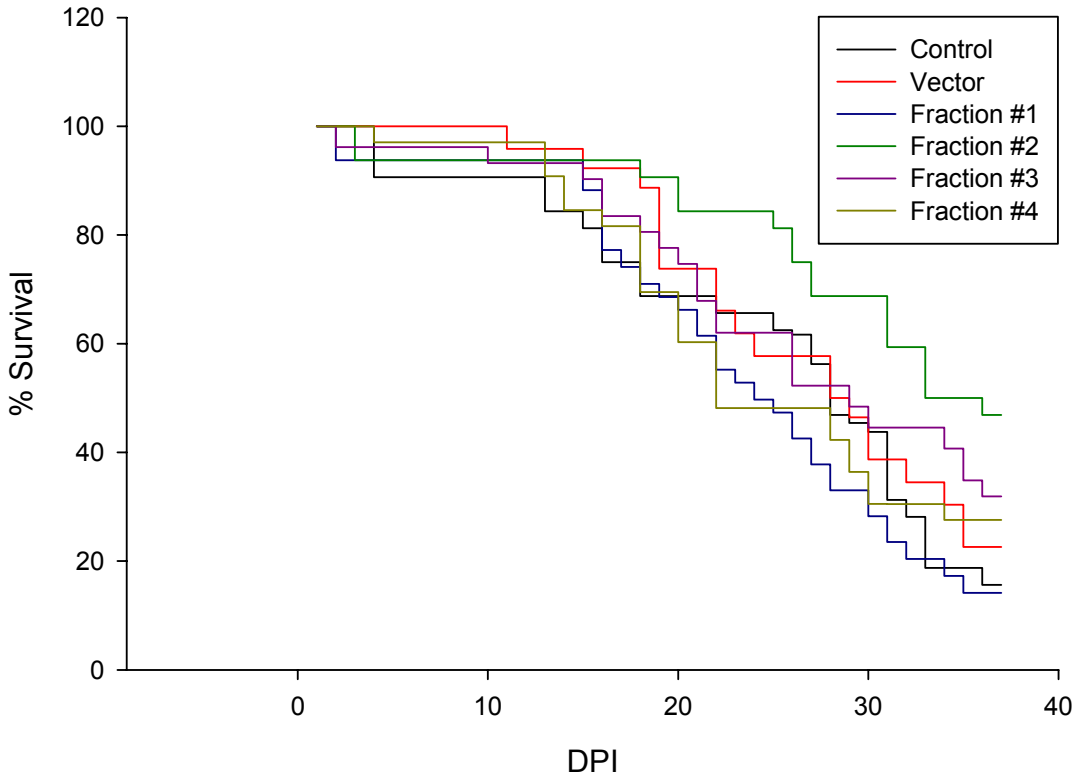

Figure 6.1. Survival curves (two tanks combined) of each of the test and control groups in AGD challenge trial 1. Fish were vaccinated twice, once at 28 and again at 14 days prior to infection, with either PBS, vector only or one of four library fractions (10 µg total DNA in 50 µl PBS) and challenged with 500 cells/L *Neoparamoeba spp*. Fish were monitored and moribund fish removed daily until 37 DPI.

Table 6.1. Number of survivors and relative percent survival (RPS) in each treatment group in each tank at the end of first AGD challenge trial.

Group	Tank 1 survivors	RPS[a]	RPS[b]	Tank 2 survivors	RPS[a]	RPS[b]
Control	1/15			2/8		
Vector only	2/12	11%		4/14	5%	
Fraction #1	2/21	3%	-9%	3/16	-8%	-14%
Fraction #2*	7/16	40%*	33%*	4/8	33%*	30%*
Fraction #3	3/17	12%	1%	6/13	28%	25%
Fraction #4	3/17	12%	1%	3/8	17%	12%

[a.] Relative percent survival vs control group
[b.] Relative percent survival vs vector only group
[*.] Indicates significantly different ($p<0.05$) from either control or vector only group in log rank test using the data from the two tanks combined.

6.3.2 Challenge Trial 2

As in trial 1 there were mortalities related to handling, water quality and non-specific bacterial infections prior to infection and again these fish were removed from analysis. Unlike trial 1 there was a significant difference ($p<0.01$) between the infection rates in both tanks. Therefore the data could not be pooled and are presented individually for the two tanks (Fig 6.2). Overall there were less moribund fish per group in tank 1 compared to tank 2 (Table 6.2). The significant tank differences resulted in different RPS for each of the groups in each of the tanks. For instance, in tank one, library fraction #2 had a significantly higher RPS than both the negative control and vector only fish (40 and 42% respectively) (Table 6.2). The split 2 group also had a significantly higher RPS at 26 and 28% compared to the control and vector only groups. The split 1 and sero-positive groups RPS's were also marginally higher. However in tank 2 the fraction #2 groups RPS was only 4 and 2% compared to control and vector only injected fish whereas all other treatment groups recorded a negative RPS when compared to both the control and vector only injected fish.

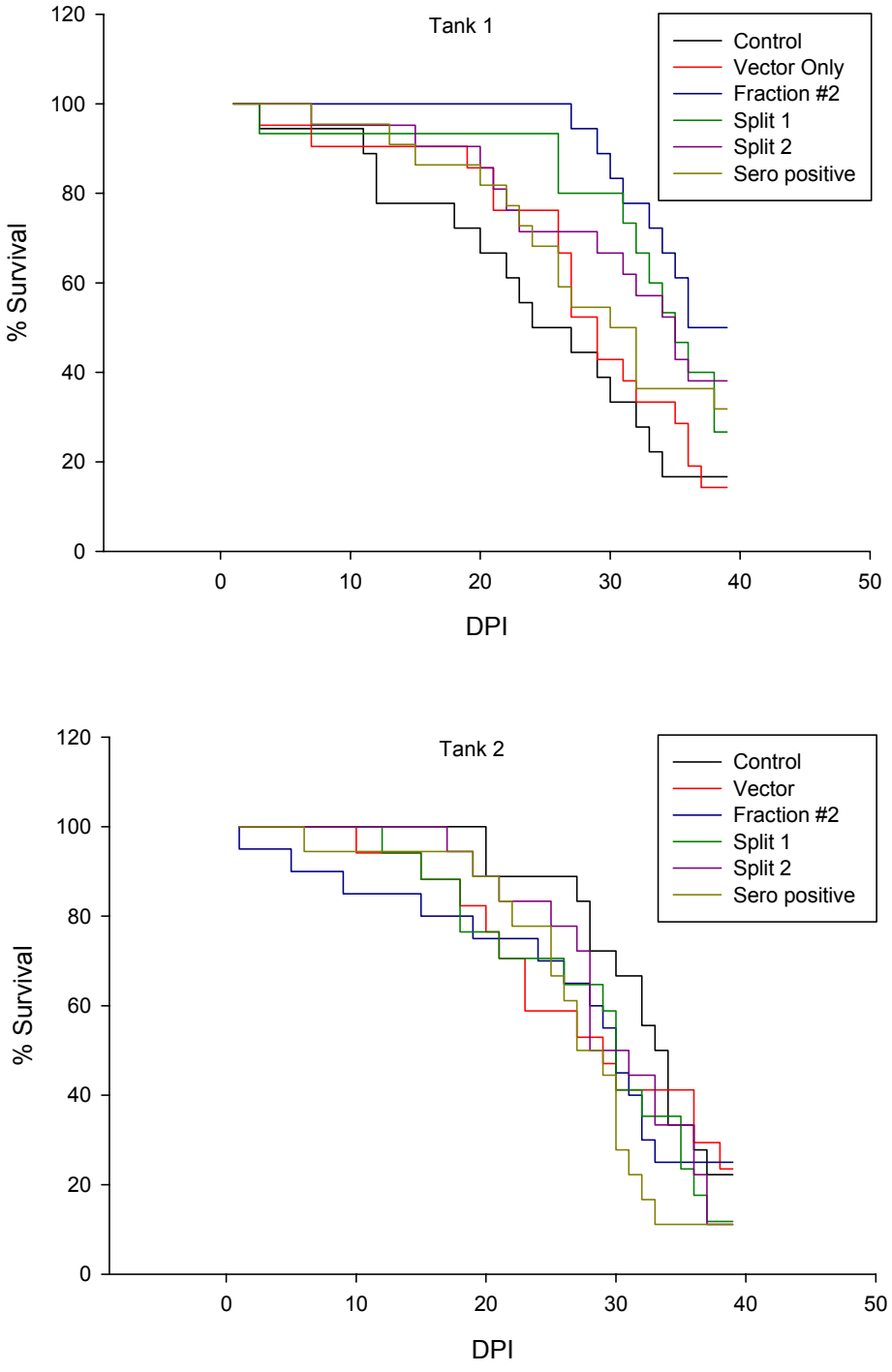

Figure 6.2. Survival curves of each of the test and control groups in AGD challenge trial 2. Fish were vaccinated at 28 and again at 14 days prior to challenge with either PBS, vector only, library fraction #2 or one of sub-fractionations of this library (10 µg total DNA in 50 µl PBS) and challenged with 500 cells/L *Neoparamoeba spp*. Fish were monitored and moribund fish removed daily until 39 DPI.

Table 6.2. Number of survivors and relative percent survival (RPS) in each treatment group in each tank at the end of first AGD challenge trial.

Group	Tank 1	RPS[a]	RPS[b]	Tank 2	RPS[a]	RPS[b]
Control	3/18			4/18		
Vector only	3/21	-3%		4/17	2%	
Fraction #2	9/18*	40%*	42%*	5/20	4%	2%
Split 1	4/15	12%	14%	2/17	-13%	-15%
Split 2	8/21*	26%*	28%*	2/18	-14%	-16%
Sero positive	7/22	18%	20%	2/18	-14%	-16%

[a.] Relative percent survival vs control group
[b.] Relative percent survival vs vector only group
[*.] Indicates significantly different ($p<0.05$) from either control or vector only group in log rank test.

6.3.3 Sequencing and Bio-informatics

Of the 288 clones sequenced from fraction #2, 183 (64%) were either empty vector, or contained small or multiple inserts. Of the remaining 105 clones, 84 consisted of good quality read greater than 150 bp. These 84 clones represented 39 unique sequences (Table 6.3). This represents an overall redundancy of about 86%. Of the 39 unique clones, ribosomal proteins accounted for 52% of the inserts and there were five inserts that returned no homology following either tblastn or tblastx analysis. In total five of the 39 unique sequences were full length and in the correct reading frame orientation. A brief summary of the 39 unique clones and their homology are presented in Table 6.3 below.

Table 6.3. Clone ID, sequence length and top tblastx hit of the 39 unique inserts sequences from library fraction #2. E-value describes the number of hits expected by chance when searching Genbank. The lower the E-value the higher the probability that the BLAST match is correct.

Clone	Size (bp)	Blast result	E-Value
P1A2	429	Zea Mays Immunophillin mRNA	E=1e-37
P1E10	519	Transcription Factor 3 (*Homo sapiens*)	E=2e-38
Multiple (7)	193	BIR Protein (*Plasmaodium*)	E= e0.68
P1G6	324	Ribosomal Protein L37 (*Drosophilla*)	E=4e-28
Multiple (4)	295	Truncated Large tumor antigen (simian virus)	E=8e-10
P1E7	204	No Significant Similarity	N/A
P1F6	709	60s Ribosomal Protein L10 (*Ixodes*)	E=7e-35
P1D4	507	Conserved Hypothetical protein (*Aspergillus nidulans*)	E=4.2e-22
P1C12	121	No Significant Similarity	N/A
Multiple (25)	394	Similar to Ac1147 (*Rattus norvegicus*) Mt protein	E=1e-04
P2C4	475	Actin (*Physarum polycephalum*)	E=1e-71
P2C12	501	Ribosomal Protein L27 (*Spodoptera frugiperda*)	E=2e-24
P2D11	889	60s Ribosomal Protein L7A (*Arabidopsis*)	E=9e-63
P2G4	467	Ribosomal Protein L22 (*Mus musculus*)	E=2e-21
P2H3	528	Ribosomal Protein S12 (*Brachiostoma*)	E=6e-36
P2F4	680	Hypothetical Protein DDB0186346 (*Dictyostelium discoideum*)	E=1e-42
P2F5	450	Ribosomal Protein S20 (*Rattus norvegicus*)	E=1e-35
P2F6	301	Ribosomal Protein L38	E=2e-05
P2F9	312	Ribosomal Protein S33	E=8e-16
Multiple (8)	219	No Significant Similarity	N/A

table 6.3 cont.

Clone	Size (bp)	Blast result	E-Value
P3H7	564	Ribosomal Protein S18 (*Ixodes ricinus*)	E=1e-27
P3A2	278	Small Sub Unit Ribosomal Protein (*C.elegans*)	E=2e-7
P3A5	541	40S Ribosomal Protein (*Perinereis aibunitensis*)	E=9e-37
P3A6	232	No Significant Similarity	N/A
P3B8	538	Ubiquitin Ribosomal Protein S27 Fusion Protein	E=4e-47
P3C8	277	Elongation Factor 1-alpha (*Leishmaniasis*)	E=3e-19
P3C9	340	Actin (*Acanthamoeba castellinii*)	E=8.3e-47
P3C12	503	Structure Specific Recognition Protein	E=0.8
P3D3	591	Ribosomal S11-1 (*Bombyx mori*)	E=1.4e-55
P3D6	504	40S Ribosomal Protein S23 (*Dermancentor variabilis*)	E=1.6e-63
P3E2	277	Ribosomal Protein L30 (*Lysiphlebus testaceipes*)	E=1.6e-26
P3E10	392	Similar to Mt Protein of unknown function	E=3e-05
P3F6	202	No Significant Similarity	N/A
P3F11	301	60s Ribosomal Protein (*Arabidopsis*)	E=6.1e-18
P3F12	277	Ribosomal Protein S8 (*Griffithsia Japonica*)	E=1.6e-0.7
P3G9	222	Putative Ribosomal Protein L9, Cytosolic (*Arabidopsis*)	E=7.1e-06
P3G11	279	40S Ribosomal Protein S8 (*Griffithsia*)	E=4e-14
Multiple (3)	301	Putative 60S Ribosomal Protein L38 (*Oryza Sativa*)	E=3e-20
Multiple (3)	440	Ribosomal Protein L31 (*Spodoptera frugiperda*)	E=1.6e-29

6.4 Discussion

Here we report on the development of an experimental vaccine able to provide a significant increase in survival for Atlantic salmon smolt challenged with *Neoparamoeba* spp., the causative agent of amoebic gill disease (AGD). The experimental vaccine comprised one fraction of an 1100 clone cDNA expression library derived from a gill-isolated amoebae complex. The average relative increase in survival was approximately 30% and 27%, respectively, when compared to non-vaccinated fish or fish vaccinated with only the vector backbone.

This is the first time that a significant increase in protection to AGD has been observed following vaccination. Although protection could be viewed as marginal the results obtained are encouraging, particularly given that AGD is caused by an external parasite, *Neoparamoeba spp*. We believe that this is the first report of a significant increase in protection in a fish species vaccinated against an external parasite. Conceivably, there is a great deal of potential in improving the protection level, just by further sub-fractionation and optimisation of the vaccination regime.

Previous attempts to vaccinate against AGD have resulted in no increase in protection despite the presence of anti-*Neoparamoeba* antibodies in the sera (Akhlaghi et al., 1996; Zilberg and Munday, 2001). In some instances the results could be due to the use of avirulent strain(s) of *Neoparamoeba* spp.. Recently it has been shown that gill derived amoebae are predominantly the newly described *Neoparamoeba perurans* whereas cultured amoebae, like the strains primarily used for vaccination, are believed to be *Neoparamoeba pemaquidensis* (Young et al., 2007). Therefore the results observed with avirulent cultures could in part be due to utilization of the wrong species of amoeba. However the use of virulent, gill derived amoebae has also not induced protection (Morrison and Nowak, 2005). Notwithstanding that the vaccination regime may be inefficient this is suggestive of either masking of protective antigens by the pathogen or that serum antibody is an ineffective defence mechanism with respect to AGD. Interestingly carbohydrate epitopes are postulated to be the immunodominant antigens of

the cell surface of gill derived *Neoparamoeba spp.* (Villavedra et al., 2005). It is possible that these carbohydrate epitopes mask or hide cell surface protein antigens that may induce an immunoprotective response. The presence of carbohydrate epitopes together with the regime of vaccinating with either whole cells or membrane preparations may skew the immune response of Atlantic salmon toward a humoral response against non-specific cell surface sugars. Indeed a screen of fish exposed either in a challenge system or naturally at sea has shown that the majority of fish respond in a non-specific manner with a characteristic 'smear' appearing in western blots (Vincent et al., 2007). This is characteristic of antibody binding to proteoglycan antigens (Fischer et al., 1996; Yeh et al., 2005). In this instance DNA vaccination offers two significant advantages. Firstly, the cDNA expression library utilised should only present protein epitopes to the host immune system, circumventing their potential masking by carbohydrate epitopes. Secondly DNA vaccination has been shown to induce both humoral (antibody) and cellular immune responses. For instance induction of CD8+ T cells and antibodies in mice following malaria circumsporozoite DNA vaccination has been found to be superior to CD8+ T cell levels induced by irradiated whole sporozoites (Doolan and Hoffman, 2002; Sedegah et al., 1994). So it is possible that the protection we observed from fraction #2 could be due to stimulation of both cellular and humoral arms of the immune system of the fish. However, further studies are needed to elucidate and confirm the mechanisms involved in the protection.

When using plasmid DNA as a vaccine it has been suggested that the presence of the plasmid DNA backbone can act as a non-specific immunostimulant. Bacterial DNA contains many unmethylated CpG motifs and these have been shown *in vitro* to be immunostimulatory in fish (Jorgensen et al., 2001; Strandskog et al., 2007). Indeed it has been reported that when used *in vivo* CpGs are able to increase resistance to AGD (Bridle et al., 2003). It has been postulated that this may occur as a result of stimulation of innate effector cells such as macrophages (Bridle et al., 2003). It is also possible that, despite using endotoxin free plasmid preparations, there are residual amounts of endotoxin present in the vaccine. Endotoxin can also act as a non-specific stimulator of an immune response. Therefore it is possible that the increase in protection observed with fraction #2

was due to non-specific immunostimulation due to extraneous components such as unmethylated CpG and endotoxin and not to the presence of *Neoparamoeba* cDNA. However, if this were the case then one would expect all fractions and the vector control to also be significantly different to control fish vaccinated with only phosphate buffered saline and not significantly different to fraction #2. On the contrary fraction #2 was significantly better than the vector only fraction, and fraction #1 in trial 1 was the same as the PBS injected group and had a negative RPS compared to the vector only group. Furthermore, fractions #3 and #4 were similar to the vector only injected fish in trial 1. This suggests that, although some non-specific protection may be occurring due to extraneous components of the vaccine, the presence of specific *Neoparamoeba* cDNA is most probably accounting for the majority of the increase in protection observed with library fraction #2.

Expression library immunisation has been used to screen for protective antigens against both bacterial and parasitic infections (Smooker et al., 2004). In many instances deconvolution to a small number of protective clones has not been possible. However, there are reports of reduction to a workable set of antigens that can be sequenced. For instance Almazan et al (2003), when using a cDNA ELI regime for tick (*Ixodes scapularis*), were able to reduce from 2705 (66 pools of 41 clones) clones to 351 clones in a secondary screen. Following sequencing this was further refined to 64 clones based on functionality of the plasmid and inserted gene, and the tertiary screen undertaken with 16 sub-pools. The results of the tertiary screen indicated that five sub-pools were efficacious in inhibiting tick infestation. A similar approach resulted in the deconvolution from a total of 30000 clones to a group with as little as five clones that confers significant protection to visceral Leishmaniasis in mice (Melby et al., 2000). Ivey et al (2003) were able to go further and deconvolute from a group of 800-1000 clones to a single clone that demonstrated protection to *Coccidioides immitis* infection. This was the first such report of deconvolution to a single clone. In our attempt to deconvolute we were unable to achieve either enhanced or similar protection compared to the primary fraction. It is possible that a number of clones obtained in the original fraction are responsible for protection and by arbitrarily dividing the library in half we diluted the protective effect.

Through sequencing we were able to identify five specific clones that, based on full length, open reading frame (ORF) and orientation analysis, would be expressed as protein in the salmon muscle. It is these clones then that possibly account for the increased protection. This will be reported in the following chapter. However, our methodology has shown that ELI in combination with sequencing and bioinformatic analysis is a powerful tool and may provide a way of developing an efficacious vaccine for AGD.

One of the cornerstones of ELI is that it attempts to screen antigens in an unbiased manner. However, when attempting to deconvolute with only a limited number of test groups, decisions need to be made on the most efficient way to refine the primary ELI fractions. Therefore, in a targeted attempt to deconvolute the protective fraction we undertook a sero-screening approach. Although this approach is entirely rational it presumes that clones invoking a humoral response are important for protection, and this approach may bias toward immunodominant antigens that are not actually immunoprotective. As discussed above the presence of serum antibodies to *Neoparamoeba* does not seem to confer protection to AGD. T cell responses have been shown to be important in defence to parasites (Smooker et al., 2004) and DNA vaccines stimulate both the humoral and cellular arms of the immune system (Donnelly et al., 1997). So in this instance T cell responses to the antigens contained in fraction #2 may be responsible for the observed increase in protection. However, there are limited reagents to study cellular responses in fish. As more reagents become available this may be a worthwhile avenue of investigation.

When attempting to deconvolute ELI fractions, sequencing of the constituent clones is a viable option especially when the library consists of a manageable number of clones. Moreover such characterization will allow compensation for any redundancy that one associates with random expression libraries. This approach was used by Almazan et al (2003) to screen their secondary pool (351 clones) and make targeted decisions on how to pool clones for their tertiary screen. This enabled them to discount non-functional (no insert) and repeat clones and genes (eg mitochondrial) which they believed would not confer protection. In doing this they were able to use 64 clones grouped into 16 pools

based on function and a further 182 clones in four pools that had no predicted function. From this they were able to identify five sub-pools that resulted in >40% efficacy toward inhibition of tick infestation and/or molting. Given that our library fraction #2 contained 288 clones we decided to sequence it in order to make decisions about how to further deconvolute. We were surprised to find that the majority (64%) of the clones contained no insert, were bad reads or contained >1 insert. This may be in part due to the use of Cre-lox to transfer the library into the expression vector. From our analysis we were able to narrow the pool down to 39 distinct clones. Further bio-informatic analysis showed that only five of these clones were full length ORF and in the correct frame. One purported advantage of the vector that we used is the ability to screen (using the HIS tag) for functional clones (Moore et al., 2001). However, given that the ATG start codon is associated with the HIS tag upstream of the insertion site, then cDNAs cloned in the wrong reading frame may still express a functional mRNA, albeit in the wrong frame. However, this may not be a true representation of the insert or any *Neoparamoeba* gene. Ideally a 3 reading frame slip vector could have been used and indeed this should be a consideration for all cDNA based ELI work. Nonetheless by performing the ELI and associated sequencing and bioinformatics we were able to narrow an 1100 clone library down to 39 clones of which we believe five are very promising and worth further consideration. The detailed work on these five potential antigenic clones is described as part of the next chapter on targeted antigen vaccination.

During both challenge trials a number of fish were lost or disregarded due to morbidity from circumstances other than AGD. Although analysis of the data demonstrated significant differences between fraction #2 and both control groups, the significance of this finding could be compromised. For instance in tank two of trial one, only eight of an original 25 fish could be considered for analysis of both the control and fraction #2 vaccinated groups. In this instance the loss of one fish results in a 12.5% decrease in percent survival. It is easy to see how small differences (for example four survivors versus two) can account for a significant difference in RPS values. In outlining standard criteria for the running of challenge experiments, Amend (1981) states that control mortality should be >60%, inter-tank variability of control mortality should be <20% and

non specific mortality during challenge <10%. In our challenges the >60% control morbidity was achieved, however there was a >20% variability in the control groups and in some instances >10% non-specific morbidity during challenge. The majority of the morbidity occurred between vaccination and challenge. This is suggestive of stress or infection occurring during this procedure. All manipulations were performed with the fish at 35 ‰ salinity, at a time when Atlantic salmon scales and skin is considered 'soft'. It is conceivable that mechanical abrasion occurred allowing non-specific bacterial infection to take hold. This is difficult to confirm as no samples were taken for microbiological analysis. The vaccine itself could have caused the mortalities, however the mortalities were not specific to any group and indeed the negative control (PBS vaccinated) was one of the worst affected. Given that there is high inter-tank variability and some non-specific losses, an increase in the number of tanks and a decrease in the number of groups per tank (ie increase in number of fish/group) was recommended for future challenges. Indeed this occurred with subsequent challenges in which an experimental design of four tanks with four groups per tank (30 fish/group/tank) was used. This will be outlined in the following chapter on targeted antigens.

6.5 Summary

In summary this chapter describes the first evidence that a vaccine for AGD may be a viable avenue worth pursuing. Given that the observed RPS was variable between tanks and relatively low, further optimisation and rationalization of the antigenic molecules was warranted. The above experiments successfully facilitated rationalization of a very crude vaccine preparation of 1100 clones, down to a potential protective antigenic fraction of 288 clones. Further, through the power of sequencing and bioinformatics we were able to achieve deconvolution of the library and have identified 39 unique contributing clones of which five warranted further investigation. However before further challenges were undertaken changes to the challenge system and protocols were considered necessary. The improved vaccination and challenge procedures along with the targeted antigen approach are outlined in Chapter 7.

7. Targeted antigen vaccination against amoebic gill disease (AGD)

7.1 Introduction

Traditionally, vaccines have been developed by cultivating the pathogen and dissecting it using either biochemical, immunological or microbiological methods in order to identify which components provide protective immunity (Rappuoli, 2001). This method, while successful in many instances, has failed to provide a means of vaccinating against numerous pathogens. Many pathogens, such as *Plasmodium falciparum*, the causative agent of malaria, are complex or are not amenable to culture. Indeed this is the case with amoebic gill disease, whereby the wild type gill isolate is infective but in all instances so far cultured *Neoparamoeba* from the gills is unable to establish AGD in naïve Atlantic salmon. In recent times the advent of genomic technologies, combined with the principles of reverse vaccination (that is, from molecular analysis to prediction of protection), has led to a paradigm shift in the way vaccines are developed. In essence, this involves using genetic information (DNA sequencing combined with bioinformatic analysis) to predict and design potential vaccines *in silico* and without the need to culture and dissect the pathogen. This has been made possible through modern high throughput genomics capabilities and large public domain genetic databases. Reverse vaccine discovery, through sequencing of *Neoparamoeba* genes and comparison with known antigens from other pathogens, particularly those of other protozoan parasites provides a very powerful approach to identify a DNA vaccine against AGD in Atlantic salmon.

In Chapter 6 we reported on our attempts to vaccinate against amoebic gill disease using expression library immunisation (ELI). ELI can be considered a ‘blind’ approach in that it attempts to achieve protection via vaccination while making no *a-priori* judgment on potential vaccine antigens. ELI in concept is diametrically opposed to that of reverse vaccination, which adopts the approach of first identifying specific molecules as potentially protective antigens. Through a combination of library fractionation (chapter 6), DNA sequencing and associated bioinformatic analysis we were able to combine the

strengths of both ELI (chapter 6) and reverse vaccination to identify a possible six unique cDNA sequences from *Neoparamoeba* spp that we considered potential candidates for a more targeted approach. One of these molecules was identified from the ELI fraction (fraction #2) that provided partial protection (see chapter 6 for details) and the remaining five from a parallel subtracted cDNA library from gill-derived *Neoparamoeba* spp (CRC project 3.4.4 and FRDC project 2002/251 Final Report). From the subtracted library we were able to identify by comparison of DNA sequences a number of cDNAs which based on their homology to either cell surface proteins and/or vaccine antigens used for other parasites (eg *Entamoeba hystolica, Plasmodium falciparum*) were deemed to be potential AGD vaccine candidates. Reported here are the details of the *in vivo* challenge trials conducted on fish vaccinated with the six target antigens.

7.2 Methods

7.2.1 Construction of antigen constructs

7.2.1.1 pbS-Sfi based constructs

Six specific *Neoparamoeba* spp. genes were identified as possible antigens from either fraction 2 of the ELI (Chapter 6) or from the subtracted library bioinformatics analysis from project 2002/251. These were designated P1A2 (327 bp), SC10 (183 bp), SN8 (468 bp), S3A4 (624 bp), S3A5 (267 bp) and S3G8 (402 bp) and all are subject of patents: in Australia as Application No. 2007221759, in USA as Application No. 11/906,440, and in Canada – Application No. 2605130. All were inserted into the pbS-Sfi vector (constructed in project 3.4.4, FRDC project 2002/251) by first amplifying the full coding sequence with primers containing the *Sfi*-A&B restriction sites. Amplified cDNA was digested with *Sfi-I* and ligated into *Sfi* digested, dephosphorylated pbS-Sfi vector. The ligation mix was used to transform electrocompetent DH10β *E. coli*. The transformed *E. coli* were plated on selective LB agar plates (10 and 100 µl) containing ampicillin (100 µg/ml) and colonies were allowed to develop overnight at 37 °C. The following day, four clones were randomly picked and grown overnight in LB medium (100 µg/ml ampicillin) at 37 °C and 200 rpm in an orbital mixer incubator. Five microliters of the overnight culture was used as template for PCR confirmation using a vector-specific forward and a

gene-specific reverse primer. Plasmid was prepared from PCR positive clones using Plasmid mini-prep kit (Qiagen) as per the manufacturers instructions and subjected to DNA sequencing and open reading frame (ORF) analysis. One positive clone from each of the six constructs was selected to make up a glycerol stock.

7.2.1.2 Confirmation of the DNA plasmid inserts by DNA sequencing

Any clone that was PCR positive (amplified an insert) was subjected to DNA sequencing. Sequencing reactions were performed in a total volume of 20 µl, containing 1 µl BigDye™ reagent (PE Applied Biosystems, Foster City, CA, USA), 3.5 µl 5X sequencing buffer (Applied Biosystems), 1 µl T7 primer (Invitrogen), 1 µl of plasmid template and 13.5 µl MilliQ water. The sequencing reactions were performed in a Master cycler epGradient S thermalcycler (Eppendorf) using the following cycling profile: initial activation for 5 min at 94 °C, followed by 25 cycles of denaturing for 10 s at 96 °C, annealing for 5 s at 50 °C, and primer extension for 4 min at 60 °C.

Prior to capillary sequencing, products were subjected to PCR clean-up using the CleanSEQ protocol (Agencourt Bioscience, Beverly, MA, USA) as per the manufacturer's recommendations. The samples were eluted with 40 µl MilliQ water, transferred into a MicroAmp optical reaction plate (Applied Biosystems, Foster City, CA, USA) and spun at 2817 x *g* for 4 s.

DNA sequencing was performed using ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequences were manually trimmed and base calls edited where appropriate. Resultant sequences were subjected to open reading frame (ORF) analysis using ORF Finder via the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/projects/gorf/).

7.2.1.3 pDEST26 based constructs

In order to facilitate shuttling of each of the six *Neoparamoeba* spp. genes between bacterial and mammalian expression vectors, they were amplified by PCR and cloned into the Gateway® entry vector pENTR (Invitrogen). Briefly, full length primers were designed for each of the six genes with the forward primer containing a CACC at the 5'

end to facilitate directional TOPO cloning. Each gene was separately amplified using PCR, and ligated into pENTR using the TOPO protocol (Invitrogen) as per the manufacturer's instructions. The *Neoparamoeba* gene-pENTR constructs were used to transform competent DH10β *E. coli*. Transformation, positive clone selection, plasmid DNA preparation and DNA sequencing were undertaken as outlined in section 7.2.1.1 and 7.2.1.2. However, instead of Ampicillin, Kanamycin was used to select for positive transformants. Once confirmation was completed, culture from one positive clone from each of the six genes was used to make a glycerol stock

Each of the six *Neoparamoeba* spp. genes were moved into the mammalian expression vector pDEST26 (Invitrogen) from pENTR using the LR clonase recombination method following the manufacturer's protocol (Invitrogen). Briefly, 5 µl of each pENTR clone plasmid DNA (150 ng) was mixed with 1 µl of pDEST26 (150 ng), 2 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and 2 µl LR clonase™ II enzyme. The reaction was incubated at 25 °C for 1 h. Following incubation, the enzyme was inactivated with 1 µl of Proteinase K solution (Invitrogen) and incubated for another 10 min at 37 °C.

Once the LR recombination procedure was completed, the clonase mixture was used to transform electrocompetent DH10β *Escherichia coli*. The transformed *E. coli* were plated on selective LB agar plates (10 and 100 µl) containing ampicillin (100 µg/ml) and colonies were allowed to develop overnight at 37 °C. The following day, four clones were randomly picked and grown overnight in LB medium (100 µg/ml ampicillin) at 37 °C and 200 rpm in an orbital mixer incubator (Ratek, Boronia, VIC, Australia). Five microliters of the overnight culture was used as template for PCR confirmation of the insert using the T7 primer (Invitrogen) and a compatible 3'gene-specific primer (GeneWorks, Hindmarsh, SA, Australia). PCR positive clones were subjected to sequencing and analysis as outlined in section 7.2.1.2. One positive clone from each of the six pDEST26-*Neaparamoeba* constructs was selected and a glycerol stock made.

7.2.2 Plasmid propagation and vaccine preparation

Plasmid constructs for all the vaccines used were prepared separately and following the same protocol. Briefly, each glycerol master stock containing the appropriate plasmid

was streaked on a pre-warmed LB/amp agar plate and grown overnight at 37°C. A single colony was picked into 5 ml of LB/Amp and allowed to grow overnight at 37°C, 500 µl was used to inoculate 500 ml of LB/Amp and the culture allowed to grow for 4 hrs at 37°C. Cells were harvested while in logarithmic phase of growth and plasmid was purified using an endo-free Megaprep kit (Qiagen, The Netherlands) as per the manufacturers instructions. Total DNA was measured using a Qubit™ fluorometer (Invitrogen) and quality checked by performing a restriction digest analysis either using *Sfi-I* for pbS-Sfi based constructs or *Sac-I* and *Mfe-I* for pDEST26 based constructs. Endotoxin content was measured using a chromogenic Limulus Amebocyte Lysate (LAL) assay kit (Lonza Australia Pty Ltd, Melbourne) as per the manufacturers instructions. Concentrated plasmid preparations were stored individually and separately at -20 °C. In order to make each of the different vaccine formulations used in the three trials, each plasmid was mixed together in phosphate buffered saline (PBS) to a final concentration of 1 µg each in a total volume of 50 µl. Control plasmids, pbS-Sfi-lox or pDEST26 were diluted to a concentration of 6µg in a total of 50 µl PBS. Once again total DNA content of each vaccine formulation was measured using a Qubit™ fluorometer (Invitrogen) and plasmid quality verified by restriction digest analysis using the appropriate enzymes for the vector. All vaccine formulations were made up less than 72 hr prior to vaccination and stored at 4 °C until used.

Fraction #2 of the expression library was prepared and stored as described in section 6.2.2.

7.2.3 Recombinant protein production

In preparation for challenge trial #3, recombinant proteins representing each of the six *Neoparamoeba* spp. genes were produced. In order to do this all six coding sequences were moved from pENTR to the bacterial expression vector pDEST17 using the LR clonase recombination method following the manufacturer's protocol (Invitrogen) as described in section 7.2.1.2. Inserts were once again confirmed by PCR using a vector and gene specific primer. PCR positive plasmids were subjected to DNA sequencing and analysis as outlined in section 7.2.1.2.

7.2.3.1 Transformation of BL21 Star (DE3)pLysS *E.coli*

Plasmids containing in-frame coding sequences were transformed into bacterial expression cells, BL21 Star (DE3)pLysS (Invitrogen). A vial of BL21 Star (DE3)pLysS One Shot cells was thawed on ice and mixed with 2 µl of plasmid DNA (100 ng). The sample was incubated on ice for 30 min, incubated at 42 °C for 30 s and immediately placed back on ice. After addition of 250 µl of pre-warmed S.O.C medium (Invitrogen), the sample was incubated at 37 °C for 1 h and 225 rpm. Two agar plates (100 µg/ml ampicillin, 34 µg/ml chloramphenicol) were plated with either 20 or 200 µl of the transformation mixture. The plates were inverted and incubated at 37 °C overnight.

A random selection of four clones was subjected to PCR analysis using a 5' vector specific primer and 3'gene specific primer. Clones with the correct insert were grown as a BL21 Star (DE3)pLysS *E.coli* stock culture in 5 ml LB medium (0.1% glucose, 100 µg/ml ampicillin, 34 µg/ml chloramphenicol) and a subculture from each stock was grown to replace the old stock once a week with an initial dilution of 1: 20.

7.2.3.2 *In vitro* translation of *Neoparamoeba* spp. antigens in *E. coli*

An aliquot of 200 µl of stock culture of each pDEST17-*Neoparamoba* gene construct was added to10 ml of LB broth (0.1% glucose, 100 µg/ml ampicillin, 34 µg/ml chloramphenical) in separate 50 ml tubes. The cultures were grown at 37°C and 200 rpm in an orbital mixer incubator for 2 h until an absorbance of 0.6 at 600nm was obtained. Following collection of a 500 µl aliquot from each culture, Isoprophyl-β-D-thiogalactopyranoside (IPTG; Promega) to a final concentration of 0.5 mM was added in order to induce protein expression. The cultures were incubated for another 2 h at 37 °C and a further 500 µl aliquot of culture collected. All samples collected before and after induction of expression were pelleted by centrifugation at 20817 x *g* for 1 min. The supernatant was discarded and the cell pellet resuspended in 100 µl of 1x SDS buffer (Promega). After heating for 5 min at 98°C, 20 µl of each sample was electrophoresed through a NuPAGE Novex Bis-Tris gel (Invitrogen) with constant 200 V for 35 min. The gel was stained overnight in coomassie staining solution (0.05% coomassie brilliant blue

R-250, 40% methanol) and then destained (40% methanol, 10% glacial acetic acid) for 2 h before images were taken using a ChemiDoc™XRS, PC gel documentation system (BioRad, Hercules, CA, USA).

For large-scale production of recombinant proteins a 250 µl aliquot of stock culture was used to seed 50 ml of bacterial media (10 ml LB broth, 0.1% glucose, 100 µg/ml ampicillin, 34 µg/ml chloramphenicol) in a 250 ml flask. These cultures were incubated at 37°C and 200 rpm for 2 h. After an absorbance of 0.6 at 600nm was obtained, 0.5 mM of IPTG (Promega) was added and cells were grown for another 2 h at 37 °C with shaking (200 rpm). The culture was spun down by centrifugation at 1699 x *g* for 20 min. The supernatant was discarded and the cell pellet was preserved and stored at -80°C until purification.

7.2.3.3 Protein purification and analysis

Protein purification was performed using a Ni-NTA Spin Kit (Qiagen, Doncaster, VIC, Australia) under denaturing conditions as per the manufacturer's recommended protocol. Briefly, the cell pellet from each 50 ml bacterial culture was resuspended in 5 ml of denaturing lysis buffer (6 M GuHCL, 0.1 M NaH_2PO_4, 0.01 M Tris·CL, pH 8.0) and incubated at room temperature for 60 min. The lysate was sonicated for 30 s (3x 10 s with 10 s interval) before it was centrifuged at 10000 x *g* for 25 min. The supernatant was applied to Ni-NTA affinity chromatography spin column (Qiagen) in sequential 600 µl aliquots, washed 3x with denaturing wash buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·CL, pH 6.3) and eluted using 3x 200 µl aliquots of denaturing elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris·HCL, pH 4.5). The protein solution was then stored at -20°C.

The concentration of protein was determined using Qubit™ fluorometer (Invitrogen). For each purified recombinant protein, a 15 µl sample was reduced by heating at 70 °C for 10 min in the presence of 7 µl reducing buffer (5 µl 4x NuPAGE LDS sample buffer; 2 µl 10x NuPAGE reducing agent) and electrophoresed through a NuPAGE Novex Bis-Tris 4-12% gel (Invitrogen) at a constant 200V for 35 min. Gels were stained (0.05% coomassie brilliant blue R-250, 40% methanol) overnight and then destained (40%

methanol, 10% glacial acetic acid) for 2 h. Bands were visualized using a ChemiDoc™XRS, PC gel documentation system (BioRad).

7.2.4 Targeted AGD Challenge Trial 1

As stated in Chapter 6, following high inter-tank variability, there was a need to increase the number of experimental tanks and number of fish per treatment group. To address this issue, the first targeted antigen trial was designed with increased tank replication (six) and sample sizes (45 fish/group). In order to accommodate the increased number of fish per group the total number of groups per tank was lowered from six to four, meaning that there were 180 fish/tank. The four groups consisted of two controls, pbS-lox control and pbS-CAT a plasmid driving the chloramphenicol acetyltransferase (CAT) reporter gene, and two test groups consisting of the fraction #2 sub-library and an equal mix of the six antigens described in 7.2.1. Due to the low number of groups the 'handling (PBS only)' control was not included. Protocols were as for the challenges outlined in Chapter 6 with one slight modification. Following acclimation, all fish were PIT tagged and vaccinated (7 µg DNA/fish). However, within 7 days of tagging and vaccination, and in the period up to 14 days post vaccination, approximately 36% of the fish across all six tanks succumbed to a combination of an ulcerative disease and non-specific bacterial infection. Therefore both on ethical and scientific grounds the trial was terminated. A replacement trial was organised and a meeting held between TSGA, TAFI and CSIRO to determine a new methodology for running the trials.

The new methodology, adopted and used in all subsequent trials apart from the replacement for the failed trial (see specific comments below), consisted of PIT tagging and vaccinating the fish – a stock all female Atlantic Salmon (*Salmo salar*) pre-smolt – in freshwater at SALTAS. Fish were PIT tagged intraperitoneally for trial #1 but intramuscularly thereafter, and then vaccinated 14 days later and received a 'boost' vaccination 14 days after the initial vaccination. Following another 14 day period fish were sorted into 4 equal groups and transported to the School of Aquaculture in Launceston. Fish were netted and moved into each of four tanks and treated immediately with 200 ml of Stresscoat (Aquasonic Pty Ltd) per tank with another treatment 48 hr

later. Fish were left to recover in freshwater for 7 days. Temperature and seawater acclimation was performed over a 14 day period and the fish were left to recover for a further 7 days. Following recovery fish were sequentially challenged with amoebae each day until a total dose of 500 amoebae/L/tank was reached. The new methodology also imposed a biomass limit of between 10 and 15 kg/tank which lowered the number of fish to approximately 120 per tank at input. However, due to the timing of the replacement trial only fish averaging 250 g could be sourced. Therefore the replacement trial consisted of 16 fish/group or 64 fish/tank. Fish were fed at a ration of 1.5% body weight per day and maintained under a 16:8 lighting regime and in a closed system with biological and mechanical filtration.

The same four treatments as in the aborted trial were used; however, due to timing issues the fish only received one vaccination. Therefore each fish received a total of 14 µg/DNA in a total volume of 100 µl for the two control groups and the six antigen group (plus CAT) and 200 µl (20 µg total DNA) for the ELI fraction #2 group. Due to the fish being PIT tagged, researchers at the staff at the University of Tasmania did not know the groups, so the trial was run until each tank recorded 70% overall morbidity as opposed to a specific morbidity percentage in the controls.

7.2.5 Targeted AGD Challenge Trial 2

Due to the low numbers of fish per treatment in trial #1 the primary purpose of this trial was to repeat the targeted antigen treatment (minus the CAT gene). Protocols for trial #2 were as outlined in section 7.2.4. Briefly, 480 fish were PIT tagged and randomly assigned to each of four groups. The control group consisted of the plasmid backbone pbS-Sfi-lox. Treatment group 1 was the six targeted antigens in pbS-Sfi. Due to difficulties of preparing large amounts of plasmid from pbS-Sfi-lox based vector, a new vector containing each of the six antigens (pDEST26, see section 7.2.1.3) was also used. A final, fourth group consisted of the six antigens in pDEST26 as well as pDEST26 driving expression of the Atlantic salmon interleukin-1β gene (Genbank accession# AY617117) as a genetic adjuvant. Fish were vaccinated 14 days after tagging with a total of 6 µg DNA in 50 µl PBS each, apart from the adjuvant group which received 7 µg

DNA. This meant that each of the vaccinated fish received 1 μg of each targeted antigen and additionally 1 μg of the IL-1β gene plasmid (adjuvant group). Fish were vaccinated again 14 days later using the same amounts and volumes as the first vaccination. Immediately prior to transport, fish were randomly assigned and divided into four tanks consisting of 30 fish/group/tank. Transport, recovery, acclimation and husbandry practices were as detailed in section 7.2.4. As previously, fish were infected sequentially with wild type gill isolate until a cumulative dose of 500 amoebae/L was reached. Fish were monitored, moribund fish were removed daily and the pit tag extracted until an overall morbidity for each tank of 70% was reached.

7.2.6 Targeted AGD Challenge Trial 3

The purpose of challenge trial #3 was to compare the efficacy of recombinant protein vaccination to DNA vaccination using the six targeted antigens. The trial once again consisted of four groups. The first was a DNA negative control (pDEST26 with no insert DNA). The second group consisted of the six *Neoparamoeba* coding sequences in pDEST 26. The third group was an equal mix of the six recombinant proteins (as prepared in section 7.2.3.4) in a 1:1 mixture of PBS and Aluminium oxide (Sigma) adjuvant. The final group was a protein control group consisting of a 1:1 mix PBS and Aluminium oxide. Protocols were as outlined in sections 7.2.4 and 7.2.5. Briefly 480 fish were pit tagged and randomly assigned to each of four groups. After 14 days fish were either injected intramuscularly with 1 μg of each DNA antigen (6 μg DNA) in a total volume of 50 μl or intraperitoneally with a total of 1 μg of each protein antigen (6 μg Protein) in 500 μl. DNA controls received 6 μg of pDEST26 whereas protein controls received 500 μl of the PBS:Aluminium oxide adjuvant mix. Fish were again vaccinated 14 days later following the same regime and allowed to rest for a further 14 days prior to transport. Immediately prior to transport fish were randomly assigned and divided into four tanks consisting of 30 fish/group/tank. Transport, recovery, acclimation and husbandry practices were as detailed in section 7.2.4 and 7.2.5. As previously, fish were infected sequentially with wild type gill isolate until a cumulative dose of 500 amoebae/L was reached. Fish were monitored, with moribund fish removed daily until an overall morbidity for each tank of 70% was reached.

7.2.7 Statistical analysis

Relative percent survival for each of the groups (compared to the negative control) was calculated using the following formula as per Amend (1981);

$$RPS = (1- (\%mortalities\ in\ vaccinated/\%mortalities\ in\ control)) \times 100$$

Each vaccine group was also compared to both the negative control and vector only control using a Cox proportional Hazards model.

7.3 Results

7.3.1 Recombinant protein production

In order to produce recombinant proteins a standard production system using the prokaryotic expression vector, pDEST17 and the *E. coli* strain BL21Star(DE3)pLysS was used. Each of the six clones was successfully moved from pENTR into pDEST17 (data not shown). Subsequent DNA sequencing confirmed that each of the six clones was in the right orientation in pDEST17 and were in-frame with the HIS fusion tag. Expression of all recombinant proteins was achieved by induction with 0.5 mM IPTG (Fig. 7.1). Recombinant proteins of the six clones of interest were successfully purified from the bacterial culture using the HIS fusion tag (Fig. 7.2).

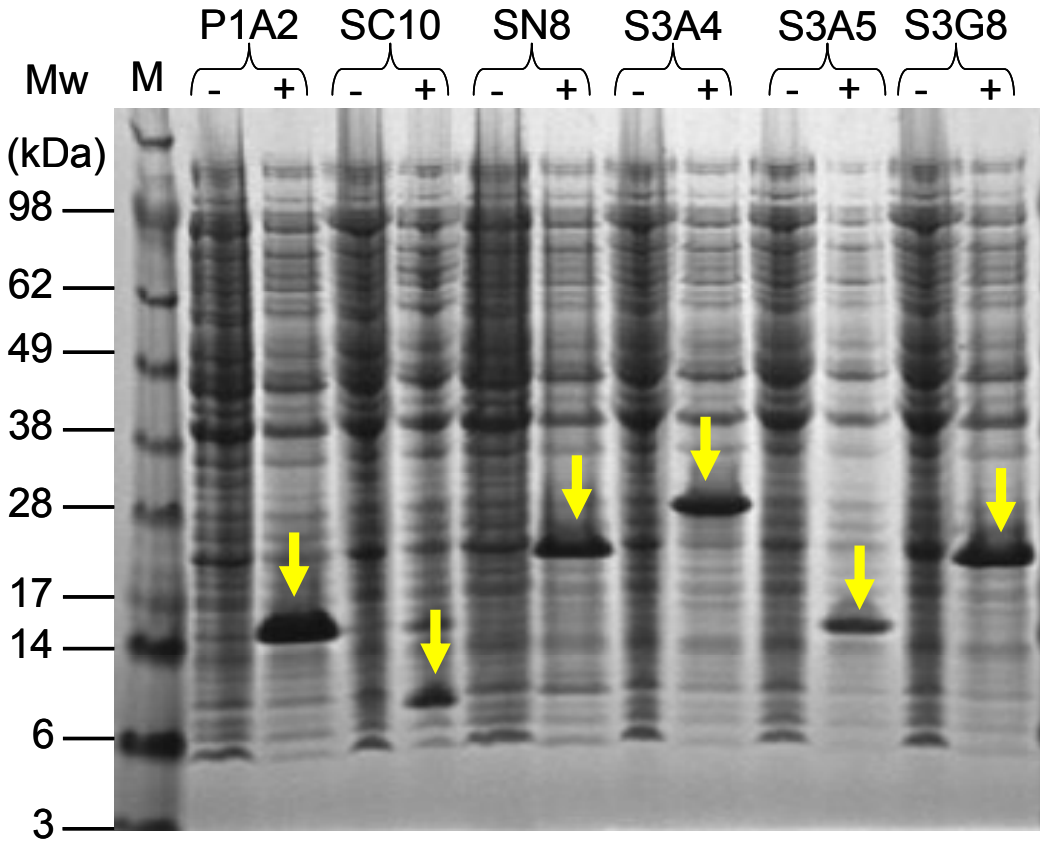

Figure 7.1. Overexpression of recombinant *Neoparamoeba* proteins by *E. coli*. Cultures of each of the six clones were either left uninduced (-) or induced with 0.5 mM IPTG (+). Five hundred microlitres of each culture was centrifuged, the supernatant discarded and the cell pellet resuspended in100 µl 1x SDS reducing buffer, boiled for 5 min at 98 °C and electrophoresed through a NuPAGE Novex Bis-Tris gel (4-12%) and stained with coomassie blue. (M) Marker (SeeBlue Plus2 Pre-Stained Standard; Invitrogen). Arrows indicate over expression of the recombinant protein.

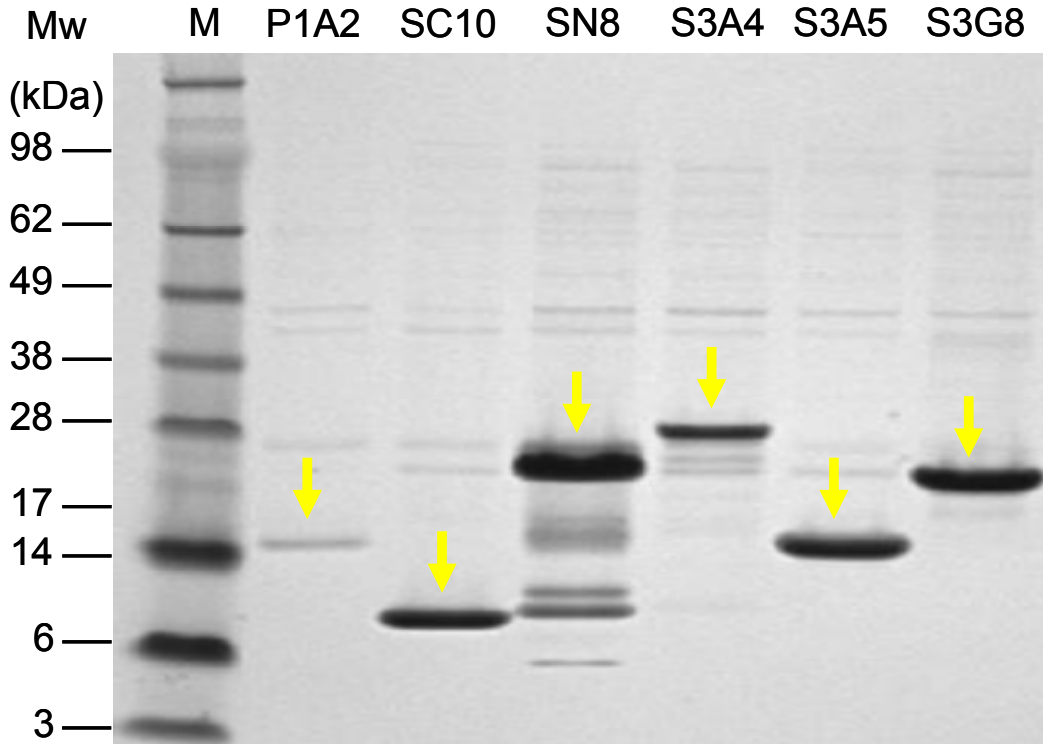

Figure 7.2. NuPAGE Novex Bis-Tris Gel (4-12%) showing the six purified HIS-tagged recombinant proteins. Each protein was purified from 50 ml bacterial culture. Bacterial cell pellets were lysed in 5 ml denaturing lysis buffer (pH 8.0), sonicated for 30 s (3x 10 s with interval) before clarification by centrifugation. Supernantants were applied to Ni-NTA affinity chromatography spin column (Qiagen) and HIS-tagged protein purified as per manufacturers instructions. Following elution, 15 µl of each purified protein was combined with 7 µl reducing buffer, denatured at 70 °C for 10 min and electrophoresed through a NuPAGE Novex Bis-Tris 4-12% gel. (M) Marker (SeeBlue Plus2 Pre-Stained Standard; Invitrogen). Arrows indicate the recombinant proteins representative of each of the six *Neoparamoeba* genes.

7.3.2 Targeted Trial 1

Three tanks were terminated at ca. 70% morbidity within an individual tank, and this was at 58, 56 and 60 days post infection (dpi) respectively and survival curves generated for each tank. Inter-tank variability was not significant (p=0.71) therefore data from each group in each tank were pooled and averaged (Fig 7.3). Only the 'selected clone' group provided a significantly greater (p=0.019) increase in protection in relation to the control group with an overall relative percent survival (RPS) ranging from 33-53% at the termination point (70% morbidity in the tank) (Table 7.1).

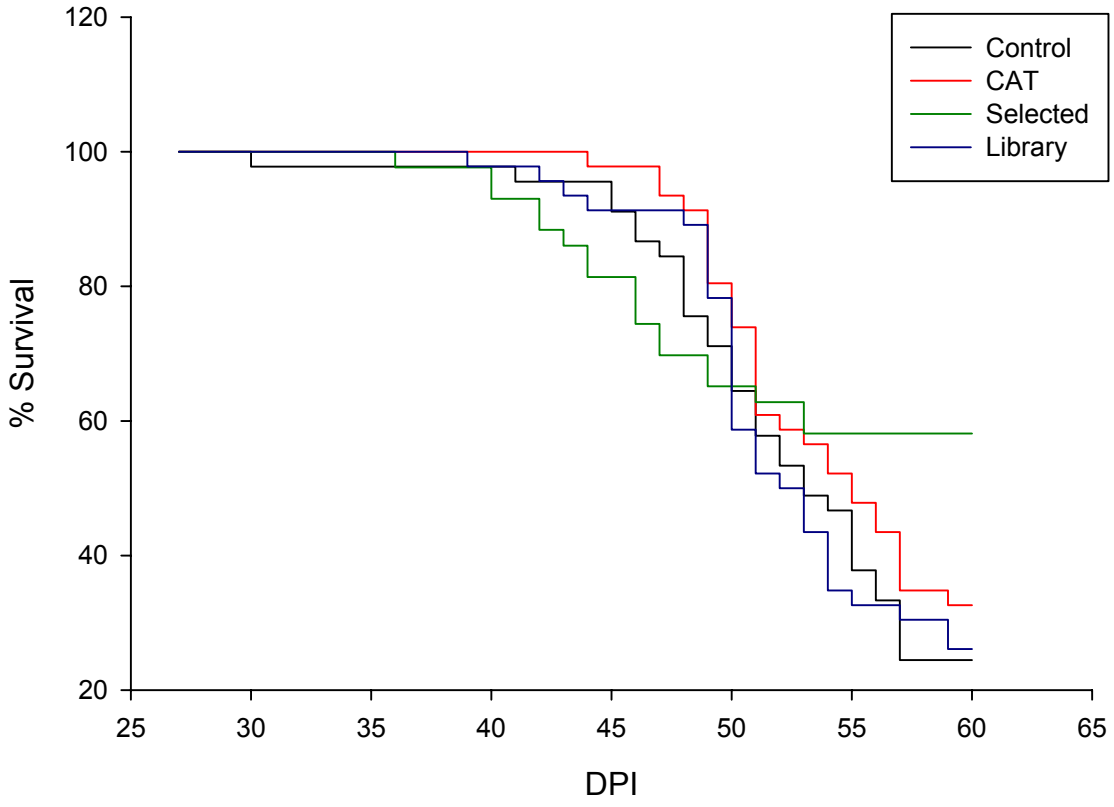

Figure 7.3. Survival curves (three tanks combined) of each of the test and control groups in targeted AGD challenge trial 1. Fish were vaccinated at -28 DPI with vector only (14 µg DNA in 100 µl), pbS-Sfi-CAT (14 µg DNA in 100 ul), six targeted antigens plus pbS-Sfi-CAT (14 µg in 100 µl) or library fraction #2 (20 µg in 200 µl) and challenged with 500 cells/L *Neoparamoeba spp* gill isolate. Fish were monitored and moribund fish removed daily until an overall tank morbidity of 70% was reached.

Table 7.1. Number of survivors in each treatment group in each tank in targeted AGD challenge trial #1.

Group	Tank 1	RPS	Tank 2	RPS	Tank 3	RPS
Control	4/17		3/12		4/16	
pbS-Sfi CAT	5/16	10%	5/14	14%	5/16	8%
Library fraction #2	5/18	6%	4/13	8%	3/15	-7%
Selected	9/14	53%*	8/13	49%*	8/16	33%*

* Indicates significantly different from both Control and pbS-Sfi-CAT

The 'library fraction' which previously provided 27% RPS (compared to vector only vaccinated controls, see Chapter 6) was this time not significantly different from the controls (Table 7.1). Most probably the reason for this was that in the new protocol, fish were only vaccinated once with a dose of 20 µg instead of receiving two doses of 10 µg. This meant that a larger than normal volume of vaccine was administered (200 µl instead of 100 µl). This larger dose resulted in a lot of 'flow back' of the vaccine making it difficult to ascertain the amount of plasmid either delivered or taken up. Given that there are 288 clones in this group then a combination of flow back and dilution of possible protective clones may have resulted in a reduced response to the possible protective clones.

The AGD infection in the fourth tank was different from the other three tanks. By 86 dpi only approximately 40 % of the fish had succumbed to AGD. Therefore, it was decided to terminate the tank and give each of the surviving fish a 'crude' gill score (visual inspection of percent of gills with AGD lesion) and to take the gills for processing and histological scoring. This scoring system was the same as that used by industry and was done by an industry-experienced person using a sliding scale of 0-5 with 0 being no observable lesions present and 5 defined as a 'heavy' fish with multiple lesions from multiple focal points. There was a wide range of gill scores covering the whole spectrum from 0 through to 5. The average gill score for the 'Selected clones' group was 1.11 and the other three groups ranged from 2-2.8 (Fig 7.4a). Histology was performed by University of Tasmania staff experienced in this process, and scores varied from an average of 23% filaments affected for the 'selected' group to 64% for the 'CAT' injected group (Fig 7.4b). Both crude gill and histology scores were subjected to analysis using

ANOVA, and while the average crude gill score and percent affected filaments for the 'Selected clones' group were significantly different from both the control and pbS-Sfi-CAT groups (p=0.028) it was not from the 'library fraction #2' vaccinated group (p=0.108). Moreover, the library group was not significantly different from the controls at the 0.05 significance level for both crude gill score and histology.

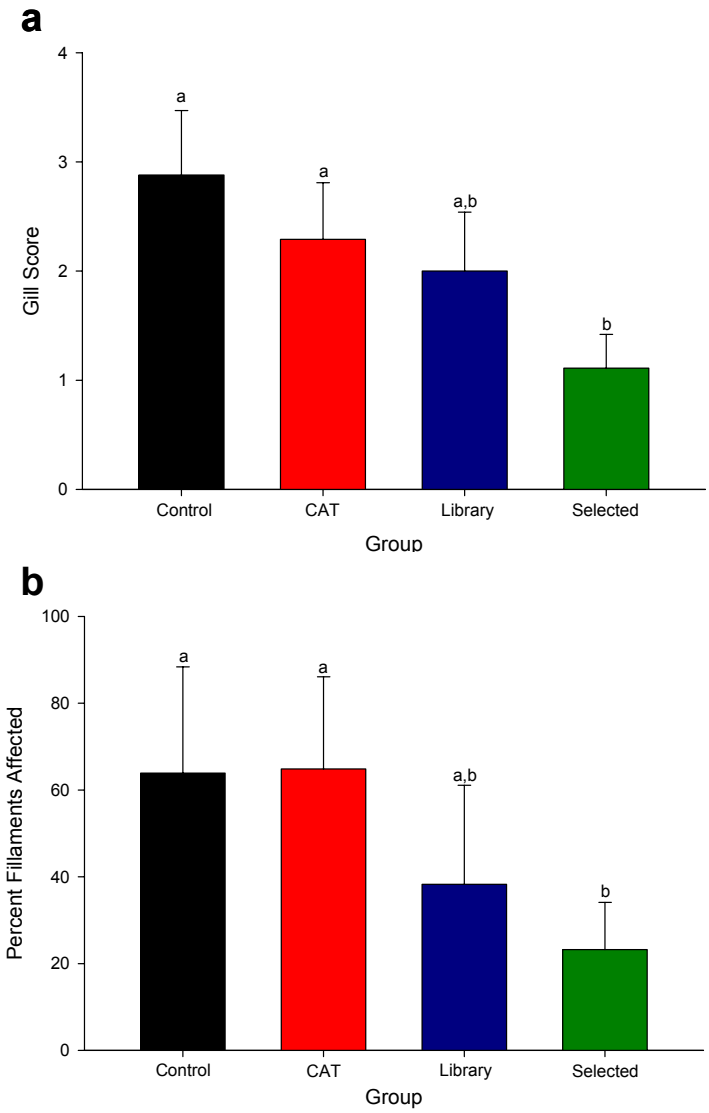

Figure 7.4. Average (±2SE) Gill scores and percent filaments affected for each of the four vaccine groups in tank C4 (a) Average crude gill scores and (b) Histology, average percent filaments affected. Superscript letters above bars indicate groups that are not significantly different from each other.

7.3.3 Targeted Trial 2

When performing the 'boost' vaccination it was noted that the fish were suffering from gas bubble syndrome due to O_2 super-saturated water. Therefore the fish were left at SALTAS for a further 14 days until this was resolved (28 days in total) before they were sorted and transported to the University of Tasmania. During temperature acclimation a mass mortality event occurred where approximately 25% of the fish died. Although the exact cause was unknown it was considered to be due to a toxicant in the incoming freshwater supply. Once mortalities subsided the fish were re-sorted across the four tanks to approximately 22-27 fish/group/tank. Instead of the original 120 fish per tank there were now approx 100 per tank. Temperature and seawater acclimation and challenge then proceeded as normal. Moribund fish were noted approximately 30 dpi and this continued until termination of each tank. Tanks were terminated on different days (Tanks C3 and C6 49 dpi, Tank C5 56 dpi and Tank C4 60 dpi) as a result of a significant difference ($p=6.15 \times 10^{-13}$) in infection rates. Despite this, morbidity in all tanks had the same trend with all of the three treatment groups having significantly better survival than the control group ($p=1.3 \times 10^{-9}$), however there was no difference between each of the treatment groups (Fig 7.5). The RPS for the pbS-antigen injected fish ranged from 27-39%, the pDEST26-antigen group ranged from 23-44% and the pDEST26-Antigen/Adjuvant group from 19-28% respectively (Table 7.2). There was no treatment by tank interaction, i.e. despite there being an overall difference between tanks there was no difference within each group across the four tanks. Overall, following hazards testing, the control fish were 2.7 times more likely to succumb to AGD and this translated to an effective increased median survival time of seven days for vaccinated fish regardless of which of the three vaccine formulations it received.

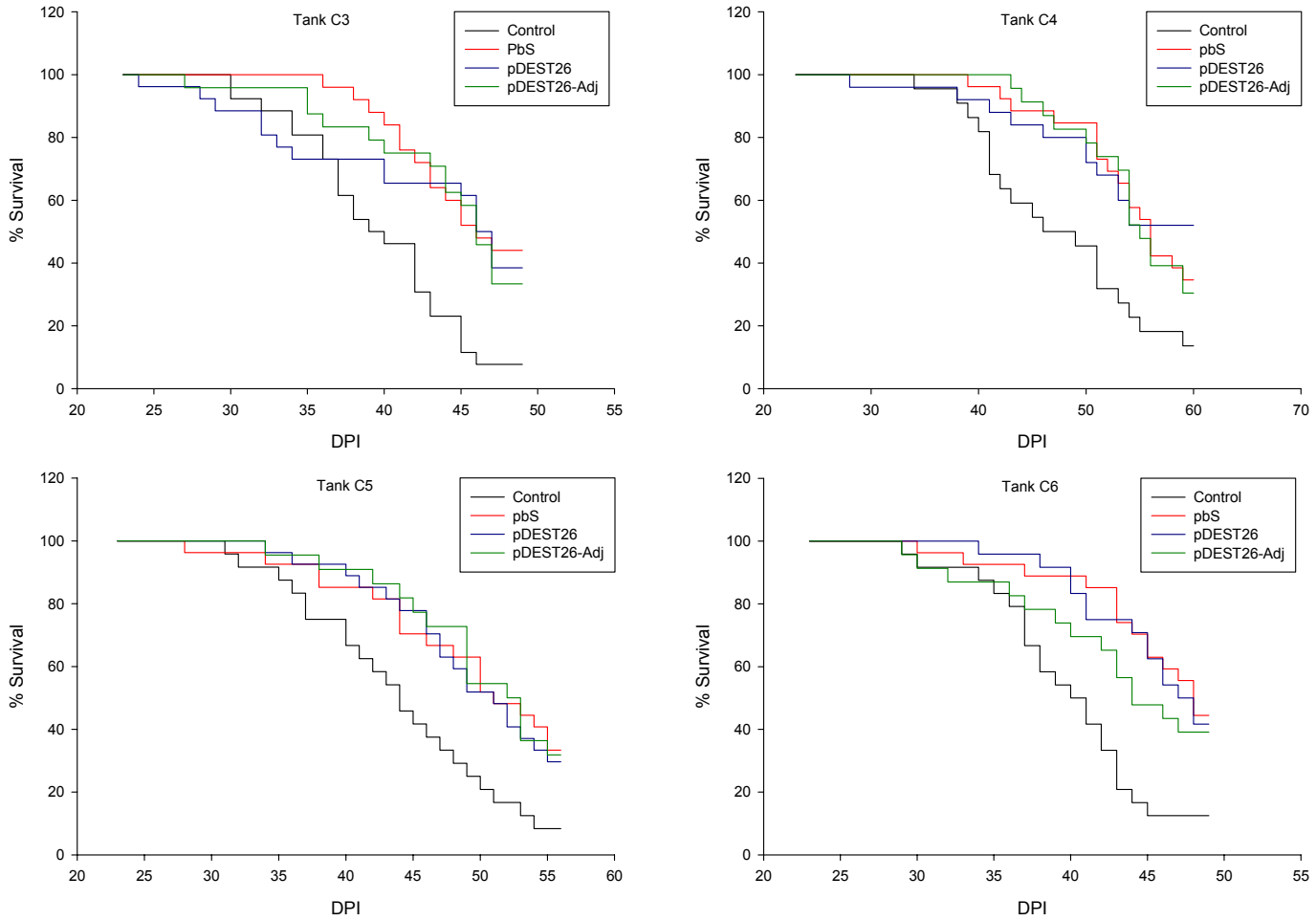

Figure 7.5. Survival curves for the 4 tanks in targeted antigen trial #2. Fish were tagged and vaccinated in freshwater, transported to UTas, saltwater and temperature acclimated and challenged with a dose of 500 cell/L. Tanks were terminated when overall morbidity reached 70%.

Table 7.2 Number of survivors in each treatment group in each tank in targeted AGD challenge trial #2

Group	C3[#]	RPS	C4[#]	RPS	C5[#]	RPS	C6[#]	RPS
Control	2/26		3/22		2/24		3/24	
pbS-Antigen	11/25	39%*	9/26	25%*	9/27	27%*	12/27	36%*
pDEST26-Antigen	10/26	33%*	13/25	44%*	8/27	23%*	10/24	33%*
pDEST26-Ant/Adj	8/24	28%*	7/23	19%*	7/22	25%*	8/22	27%*

*Indicates significantly different to control vaccinated fish
[#]Refers to tanks C3, C4, C5 and C6 respectively

7.3.4 Targeted Trial 3

Targeted trial #3 was undertaken in a newly-built tank system at the University of Tasmania and not in the same system as the previous two trials. Soon after challenge (6 dpi) the heater/chiller unit in tank 1 failed. All fish were subsequently moved to a separate 'clean' tank and once the unit was repaired they were relocated into their original tank (14 dpi) (but no additional amoebae added). Due to mechanical abrasion and stress as a result of handling, fish in this tank started to succumb to skin lesions and non-specific bacterial infection. Therefore, in consultation with Phil Crosbie from the University of Tasmania it was agreed to lower the temperature in this tank to 12 °C at 27 dpi. The non-specific mortalities continued to occur up to 40 dpi. By this time some 24 fish were lost to this condition (25% of the population). Similarly some fish in tanks 2 and 3 also displayed skin lesions and non-specific bacterial disease that resulted in some losses (2 fish in tank 2 and 12 fish in tank 3). To alleviate this, the temperature in these tanks was also lowered to 12 °C at 27 dpi. When the incidence of skin lesions had subsided (53 dpi) the temperature in these three tanks was again raised back to 15 °C. Due to the manipulations described, AGD progressed differently in each tank. Tank 4 (the only tank that was not altered during the challenge) was terminated at 49 dpi (70% overall morbidity). Tanks 1, 2 and 3 were terminated at 85 dpi. Tanks 1 and 4 had reached the predetermined cut-off of 70% overall morbidity however tanks 2 and 3 had only reached 61% and 54% respectively. The decision to terminate tanks 2 and 3 prior to the pre-determined cut off point was taken as the disease progression had reached a plateau and time was limiting. The above factors all contributed to a significant tank to

tank variation (Fig 7.6). Furthermore, there was a significant tank by group interaction, i.e. the same groups performed differently across tanks. Therefore data from all tanks are presented separately. Survival of the DNA injected group (pDEST26-6 clones) was significantly higher than DNA control injected fish in tanks 1, 2 and 4 (RPS 38%, 30% and 41% respectively) but not for tank 3 (-8%) (Table 7.3). The relative percent survival for the protein vaccinated group compared to the adjuvant only controls was significantly lower in tanks 1, 2 and 3 (-45%, -42% and -26% respectively) (Table 7.3). However, survival was significantly higher in tank 4 (RPS 32%), the only tank in which the temperature was not altered during the challenge.

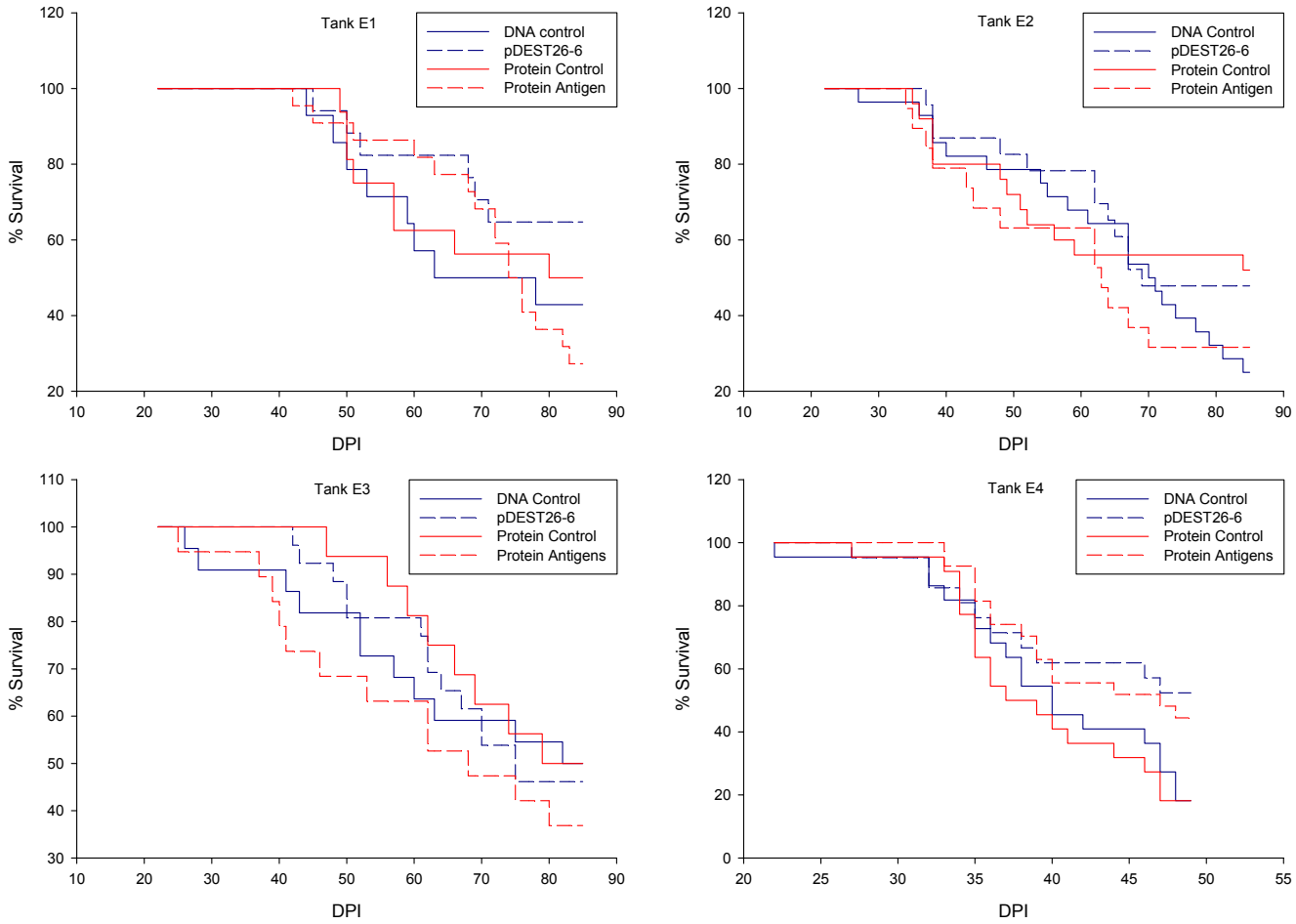

Figure 7.6. Survival curves for the four tanks in targeted antigen trial #3. Fish were tagged and vaccinated in freshwater, transported to UTas, saltwater and temperature acclimated and challenged with a dose of 500 cell/L. Tanks were terminated when overall morbidity reached 70% apart from E2 and E3. Solid lines are the controls and dashed lines are the two test groups. DNA groups are blue and protein groups are red.

Table 7.3 Number of survivors of each treatment group in each tank in targeted AGD challenge trial #3

Group	E1	RPS	E2	RPS	E3	RPS	E4	RPS
DNA Control	6/14		7/28		11/22		5/23	
pDEST26-6	11/17	38%[a]	11/23	30%[a]	12/26	-8%	11/21	39%[a]
Protein Control	8/16		13/25		8/16		4/22	
Six Proteins	6/22	-45%[b]	6/19	-24%[b]	7/19	-26%[b]	12/27	32%[c]

[a] indicates survival is significantly higher than DNA control group
[b] indicates survival is significantly lower than protein control group
[c] indicates survival is significantly higher than protein control group

7.4 Discussion

Here we report that a combination of six antigen DNA vaccine is able to provide a relative increase in protection of 37% on average when compared with fish injected with DNA vector alone. This increase is similar using either a homologous promoter (Atlantic salmon β-actin) or a heterologous promoter (CMV) to drive foreign gene expression in Atlantic salmon muscle cells. Furthermore, there is some evidence that in a more chronic infection the six antigen DNA vaccine results in significantly lower crude gill scores and percentage of filaments affected in AGD challenged fish. At this stage under the regime used, it appears as though vaccination using bacterially derived recombinant proteins representative of the six *Neoparamoeba* genes in combination with Aluminium Oxide adjuvant is unable to increase protection over the adjuvant alone.

Currently there are no other reports of successful traditional or DNA vaccination attempts against external parasites in fish. Therefore a true comparison of the RPS obtained is difficult. However, compared to other DNA vaccination studies (namely against viruses) in fish the RPS of 37% reported here is comparatively low. For instance DNA vaccines using the G-protein from either IHNV or VHSV provide RPS of >90% (Lorenzen, 1999; Winton, 1997). However, lower RPS of between 33 and 48% has been reported for other viral diseases such as spring viraemia of carp virus (SVCV) (Kanellos et al., 2006). Likewise DNA vaccination against bacteria, either *Aeromonas veronii* or *Vibrio anguillarum,* in spotted sandbass (*Paralabrax maculatofasciatus*) or Asian seabass (*Lates*

calcarifer) achieves RPS of between 53-61% and 55% respectively (Kumar et al., 2007; Vazquez-Juarez et al., 2005). Notwithstanding the inherent differences between viruses, bacteria and parasites and the obvious differences in host response a number of explanations may account for the low RPS we observed. For instance it could be due to the timing of vaccination. In all instances the fish were vaccinated while undergoing smoltification. Smoltification is a stressful procedure and often results in a depression of the immune response. Another reason for the relatively low RPS could be the vectors used. Both pbS-Sfi and pDEST26 are N-terminal HIS tag fusion vectors. Interestingly when vaccinating against SVCV, fusion vectors using either HIS or lacZ fusion tags only resulted in 3% RPS whereas the corresponding vectors without a fusion tag resulted in an RPS of 48% (Kanellos et al., 2006). Finally our six antigen vaccine can be considered to be quite crude as we were unable to undertake refinement of either the formulation or the dose. Therefore, there is conceivably considerable scope to improve the RPS achieved against AGD either by refining the dose, the makeup of the vaccine, timing of vaccination or even by changing the delivery vector used. This however requires significant further effort.

In an attempt to increase the RPS of our six antigen vaccine formulation we tried co-administering plasmid DNA with the CMV promoter driving Atlantic salmon IL1-β expression as a possible adjuvant. IL1-β is a cytokine and cytokines have been evaluated as possible adjuvants in mammalian DNA vaccination studies (Greenland and Letvin, 2007). In general cytokines regulate immunological responses by recruiting and stimulating T cells, or by acting directly on infected cells (Barouch et al., 2004). For instance, IL-2 induces the proliferation and activation of T cells and has been shown to act as an adjuvant to plasmid DNA vaccination in multiple animal models (Barouch et al., 2004). Murine Granulocyte-macrophage colony-stimulating factor (mGM-CSF) has been used as a genetic adjuvant when testing a DNA based vaccine against SVCV (Kanellos et al., 2006). Plasmid GM-CSF has been shown to recruit both macrophages and dendritic cells to the site of inoculation (McKay et al., 2004). It has also been shown to augment vaccine-elicited antibody and $CD4^+$ Tcell responses (Barouch et al., 2002; Xiang et al., 1995). We chose IL-1β as it is a pro-inflammatory cytokine and it acts to mediate the

immune response to a variety of pathogens (Dinarello, 1996). IL-1β has also been shown to be up-regulated in natural AGD infection (Bridle et al., 2006) and is implicated as being a mediator of the response to AGD (Bridle et al., 2006). Unfortunately, IL-1β co-administration was not able to increase the RPS of the vaccine. Indeed, the RPS was slightly but not significantly lower than the vaccine alone. It could be that IL-1β is non-protective and or non-immunostimulatory or that the timing of co-administering IL-1β was wrong. Unfortunately we were unable to have an IL-1β control group to ascertain if it was immunostimulatory or protective alone. If the exact mechanism of protection provided by the six antigen vaccine is elucidated then it may be possible to incorporate the appropriate cytokine adjuvant or another DNA vaccine adjuvant such as CpGs. This however was beyond the scope of this project and requires a significant research effort.

During the course of the challenge trials we switched from our original vector, pbS-Sfi to the commercially available pDEST26. The reason for this switch was based on technical difficulties in preparing enough of the pbS-Sfi based plasmids. pbS-Sfi was built using the Atlantic salmon β-actin promoter to drive expression whereas pDEST26 uses the CMV promoter. We originally decided to use the β-actin promoter as we had shown that it resulted in increased expression of the CAT reporter gene in Atlantic salmon muscle (see Final Report for project 3.4.4;2002/251). Another reason for initially choosing the β-actin promoter based plasmid is that there are perceived regulatory concerns regarding the use of CMV based plasmids in aquaculture (Hallerman and Kapuscinski, 1995). CMV promoter is derived from a human pathogen and while CMV is known to integrate into genomic DNA there is no evidence that the promoter integrates when used in plasmid DNA. Furthermore, it should be noted that a CMV based DNA vaccine for IHNV is commercially available for use in North America (Hensley, 2005). Despite the ability of the Atlantic salmon β-actin promoter to drive increased expression of CAT, we do not know if it increased expression of any of the six antigens. Furthermore, response to DNA vaccination is also reliant on successful translation of the mRNA to protein and in turn the generation of an immune response. Again this has not been measured. Despite these potential differences there was no difference in the protection levels achieved with either the β-actin or CMV based vectors, indicating that increased expression may not result in

an enhanced response. However, it may be worthwhile constructing a new β-actin based vector that is more amenable to large scale plasmid DNA preparation. Further studies comparing β-actin and CMV expression, protein translation and immune response are also warranted.

Despite a significant relative increase in survival of vaccinated groups in all trials so far, surviving fish vaccinated with the six antigen vaccine still present with clinical AGD (albeit with varying degrees of severity). In commercial production, AGD is managed by routine bathing of the fish in freshwater. This intervention is applied prior to fish becoming moribund and is based on deriving an average 'gill score' on a sub-sample of fish taken from a cage. Therefore the use of a challenge model that uses morbidity or survivability rather than gill scores as an endpoint may be of less practical use. Indeed, unless there is a positive link between 'gill score' and time to morbidity then it would prove difficult to translate the results obtained from our vaccine trials to the field. However, recent evidence from field based experiments has shown that there is a direct correlation between the gill score of a fish and its subsequent survivability if left untreated (R Taylor, CSIRO, personal communication). In trial #1 we were able to perform both crude gill scores and histological analysis of a number of infected fish that survived past termination of the experiment. Notwithstanding that this was only performed in one tank and that the numbers of fish per group were low (c.a. 9 fish/group), fish vaccinated with the six antigen vaccine demonstrated significantly lower gill scores and percentage of filaments affected. Indeed, on the farm a cage with an average score of 1.11 has not yet reached the trigger point for bathing whereas a cage with an average of 2 or greater has reached the trigger for bathing. Therefore, the fish vaccinated with the six antigen DNA vaccine could conceivably require less frequent baths in a production cycle. This is encouraging given that the RPS provided by this vaccine is low compared to DNA vaccination against viral diseases. Hence by reducing the number of baths over a production cycle there still may be a positive effect by using the '6-antigen crude' vaccine with regard to managing AGD. However, the only real way to test this is to vaccinate fish and subject them to 'natural' infection in the field.

In trial #3, in all but one tank, vaccination with a combination of six recombinant proteins representative of the six DNA antigens failed to provide protection. Indeed, the RPS for protein vaccinated fish was negative compared to adjuvant only injected fish. Interestingly, the only tank in this trial to show a positive RPS (32%) was the one tank in which no manipulations of environmental conditions were undertaken and no non-specific mortalities occurred. It could be argued that these manipulations affected the course of infection in the three other tanks. This however is unknown and can only be tested by re-running the experiment. Despite this, there may be a number of practical reasons why the recombinant proteins failed to provide protection. Firstly, the proteins were made in a bacterial system which means that unlike the natural *Neoparamoeba* proteins they may not undergo post-translational modifications. These modifications may be important for such things as protein folding and in turn exposure of the correct antigenic determinants to the immune system. Theoretically, post translational modification would occur to proteins expressed in the fish following DNA vaccination, since the fish cells are eukaryotic. Secondly, an incorrect dose may have been used. A dose of 1 μg of each protein was used based on other protein vaccinations in fish. However, this may be too little protein to induce a proper response. Unfortunately we did not sample fish to monitor their antibody response, as this was would introduce extra handling and increase the likelihood of loosing fish to non-specific infections. Finally, protein vaccination skews the response toward the humoral side of the immune system and the generation of antibodies. As discussed in Chapter 6 the humoral response to AGD may be an inappropriate mechanism for protection. Therefore, failure of the recombinant protein version of the vaccine to increase protection may be due to a number of factors. The positive result in one tank is encouraging and may warrant further work in this area using recombinant proteins produced in a eukaryotic system, different dosage rates and different adjuvants to help skew the immune response appropriately. Use of a protein vaccine will circumvent any potential regulatory limitations that may be associated with DNA vaccination.

In all three trials reported here there were significant tank differences. In trial 1 this was due to the infection not running its course in one tank (no difference between the other 3

tanks). In trial 2 the significant differences were likely due to different rates of disease progression in each of the tanks to reach the pre-determined overall 70% morbidity cut off. In trial 3 it was in most part due to manipulations of temperature in the tanks. In trials 1 and 2 there was no interaction between tank and group effects however in trial 3 there was. An interaction between tanks and treatments is a concern as it decreases confidence in the results obtained. Despite differences between tanks in each trial, results for fish injected with the six antigen vaccine were reasonably consistent, with the group displaying an RPS between 23 and 53%. One tank in trial 3 had a RPS of -8% but it is difficult to ascertain how the environmental manipulations, such as lowering the temperature or termination prior to the pre-determined 70% overall morbidity cut off, influenced this result. The AGD challenge system has been co-evolving with our vaccine testing. Some variability is inherent in challenge systems. This variability is possibly magnified by using crude gill isolate to induce infection. Currently, no axenic culture of infective *Neoparamoeba* exists. Often with virus or bacterial model infections fish are injected with a known amount of virus particles or colony forming units (CFU) whereas the amoeba are added directly to the water. So it is difficult to determine whether each fish encounters amoebae or that the mix is uniform across the tank. We are also using outbred lines of fish in the experiments, so fish to fish variation can magnify both the between-group and the between-tank variability. The numbers of fish in each group were increased in an attempt to decrease this variability. There also may be some logistical issues that need to be sorted out with regard to this system for vaccine testing, the most pressing being the relatively high loss of fish due to unknown events, equipment failure and non-specific infections. Nevertheless, this has highlighted the need to do both spatial and temporal replication with regard to vaccine testing and AGD (as with most vaccine testing). It appears from our data that a minimum of four tanks should be used in a vaccine trial and that a trial should as a minimum be repeated to increase the confidence in the results obtained. Finally, given the potential for non-specific losses of fish from the groups a minimum of 30 fish per group per tank will be necessary.

7.5 Summary

Here we report the results of three vaccination trials using a set of 6 target antigen molecules that were identified by a combination of ELI and reverse vaccination approaches. The results show that the six antigen DNA vaccine provides a relative increase in protection of approximately 40% when Atlantic salmon are challenged with AGD. We also provide evidence that the vaccine can reduce the percentage of AGD-affected gill filaments in surviving fish following a more chronic infection. At this stage, results from the one limited trial indicate that bacterially-derived recombinant proteins do not increase protection against AGD. However, further investigation is warranted. Due to tank to tank variations in infection rates, multiple spatial and temporal replication when performing AGD vaccine experiments in the laboratory system is recommended. Moving forward, it needs to be determined how the encouraging results obtained in the laboratory system (with an acute to morbidity challenge) translate to the field where a more chronic sustained infection occurs. It is likely there remains scope to refine the vaccine in terms of its constitution, dose, delivery schedules and the possible incorporation of alternative adjuvants.

8. Molecular responses of Atlantic salmon injected with a six antigen DNA vaccine against amoebic gill disease (AGD)

8.1 Introduction

DNA vaccination has changed the way in which antigens are delivered to invoke a protective immune response. Rather than delivering attenuated forms of the pathogen or sub units, and relying on the immune system to process, recognise and respond, DNA vaccination (or immunisation) aims to directly express components of the pathogen in the host's cells (Donnelly et al., 1997). This results in continued and sustained delivery of the antigen. In order for this to happen and for the subsequent response to occur there are four key steps that need to take place. Firstly the delivered DNA needs to be taken up by the host's cells. In the case of fish this occurs in myocytes immediately surrounding the injection tract (Anderson et al., 1996). Secondly the encoded gene(s) needs to be transcribed and expressed as mRNA. Thirdly, this transcribed mRNA needs to be translated into protein. Finally, the 'foreign' protein needs to be processed and presented to the immune system so that a response can be generated. This should then result in a specific response and significant immunological memory.

In the previous two chapters we have outlined our approach to testing possible DNA vaccine candidates for AGD. It appears that we have a six-antigen formulation that provides a relative increase in survivability of ca 40% when compared to fish injected with just the delivery vector alone. However, the process by which this protection occurs is as yet unknown. Although there remain many questions regarding refining the vaccine, it was necessary to explore if one or more of the six antigens are involved in eliciting a specific immune response and hence protection. To this end an assessment was made whether all or any of the four processes – uptake, transcription, translation recognition and response - outlined above are occurring in fish vaccinated with any or all of the six antigens. Presented here in this chapter are the preliminary results on select molecular responses following vaccination by each of the six antigens. The work described was part of Ms Vivian Lim's Masters Degree Thesis (University of Tasmania) conducted at the

CSIRO Hobart laboratories. Vivian was co-supervised by Dr Richard Morrison and Dr Mathew Cook. Richard's role in providing academic supervision to Vivian is duly noted by the Authors of this report.

8.2 Methods

8.2.1 Assessment of plasmid DNA transcription *in vivo*

8.2.1.1 Preparation of plasmids (pDEST26 and pbS-Sfi)

The plasmid DNA used for the experiments was prepared as outlined in Chapter 7. Plasmid DNA was resuspended to a concentration of 20 μg/100 μl in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4), together with 0.1% alcian blue. Each plasmid was prepared separately or as a mixture with equal volume mixed, providing seven treatments (Table 8.1). A positive control vector, pDEST26 (Invitrogen, Carlsbad, CA, USA), containing S3A4 DNA was incorporated into the trial (Table 8.1). pDEST26 was chosen as it uses the CMV promoter to drive expression of HIS-tagged genes.

Table 8.1. List of vaccine constructs used in each different trial for the assessment of *in vivo* transcription.

Groups	Vaccine construct (20 μg/100 μl PBS)
Treatment 1	pbS-Sfi-P1A2
Treatment 2	pbS-Sfi-SC10
Treatment 3	pbS-Sfi-SN8
Treatment 4	pbS-Sfi-S3A4
Treatment 5	pbS-Sfi-S3A5
Treatment 6	pbS-Sfi-S3G8
Treatment 7	Equal volume mixed of each of the 6 antigens
Positive control	pDEST26-S3A4

8.2.1.2 Fish

Immature, diploid Atlantic salmon (*Salmo salar*), average weight 250 g, were obtained from Salmon Enterprises of Tasmania (SALTAS), Wayatinah, Tasmania. Fish were transferred to the CSIRO Marine and Atmospheric Research laboratories at Hobart, Tasmania, and kept in a recirculated fresh water system equipped with biological filtration. Fish were fed with commercial 6 mm extruded pellets (Skretting, Cambridge, TAS, Australia) at a ration of 2% body weight per day, kept under a 16:8 lighting regime and maintained at 16 °C. These fish were reared in fresh water at all times and therefore had never been exposed to the *Neoparamoeba* spp., which is an obligate marine organism.

In order to assess foreign gene expression in vaccinated Atlantic salmon, seven fish were injected intramuscularly with either pDEST26-S3A4, pbS-Sfi-P1A2, pbS-Sfi-SC10, pbS-Sfi-SN8, pbS-Sfi-S3A4, pbS-Sfi-S3A5 and pbS-Sfi-S3G8 separately, and one fish was injected with an equal mixture of all the above except pDEST26-S3A4 (Table 8.1). Each of the fish was fin clipped for identification. Plasmid DNA was injected into the fish muscle tissue under the dorsal fin in two injections (10 µg/50 µl x 2), both on the same side of the fish in very close proximity, using 300 µl syringes fitted with 27G needles.

At one week post-injection (PI), the fish were euthanised by an over-dose of AQUI-S (Aqui-S, Lower Hutt, New Zealand). From each individual fish, muscle tissue immediately surrounding the injected site (identified by the alcian blue stained needle tracts) and muscle tissue from a non-injected site on the same fish were collected separately, fixed in RNAlater (Ambion, Austin, TX, USA) and stored at -80°C.

8.2.1.3 RNA extraction and analysis

RNA was extracted using a RiboPure kit (Ambion) following the manufacturer's protocol. Briefly, samples were frozen in liquid nitrogen, ground to powder and then homogenized in TRI Reagent (Ambion) by passing the solution through a 19G needle and 3 ml syringe 10 times. Then, 200 µl of chloroform was added and vortexed immediately for 15 s. The samples were incubated at room temperature for 5 min and centrifuged at 12000 x *g* for 10 min at 4°C. A volume of 400 µl from the aqueous top

layer was then mixed with 200 µl of 100% ethanol and vortexed immediately for 5 s to avoid RNA precipitation. The samples were transferred into a filter cartridge-collection tube and centrifuged at 12000 x *g* for 30 s. After washing with 500 µl wash solution (Ambion) and centrifuging at 12000 x *g* for 30 s twice, 100 µl of elution buffer (Ambion) was added onto the filter column, left at room temperature for 2 min and centrifuged at 12000 x *g* for 30 s to elute the RNA.

Each RNA sample was subjected to DNase treatment by mixing 50 µl RNA sample with 5 µl of 10x DNase Buffer (Ambion) and 4 µl of DNase (Ambion). After the sample was incubated at 37 °C for 30 min, 10 µl of DNase inactivation reagent (Ambion) was added. The sample was incubated at room temperature for 2 min, centrifuged at 20817 x *g* for 1 min and the supernatant was transferred into a new RNase-free tube for storage at -80 °C.

The concentration of RNA was estimated using a Qubit™ fluorometer (Invitrogen), and the quality of RNA was assessed by 1% TAE agarose gel electrophoresis. A mixture of 8 µl of sample and 2 µl of RNA loading dye was applied to each well.

8.2.1.4 First strand cDNA synthesis

First strand cDNA was synthesised using Superscript™ II reverse transcriptase (RT; Invitrogen) following the manufacturer's protocol. An RT treatment and a non-RT control were set up for each sample. Both of them were treated similarly except that Superscript™ II RT was omitted in the non-RT control sample. A mixture of 10 µl RNA, 1 µl oligo(dT) (50 µM) and 1 µl dNTP (10 mM) was incubated at 65 °C for 5 min then on ice for 2 min. Following this, 4 µl 5X first strand buffer (Invitrogen), 2 µl DTT (Invitrogen) and 1 µl SuperaseIn™ (Ambion) were added and incubated at 42 °C for 2 min. Then, 1 µl of Superscript™ II RNase-free Reverse Transcriptase (Invitrogen) was added into the RT treatment sample while the non-RT sample received 1 µl of RNAse-free water. Following incubation at 42 °C for 50 min and 70°C for 15 min, 1 µl of *E. coli* RNase H (Invitrogen) was added to all samples. After a final incubation at 37 °C for 20 min, the cDNA products were stored at -20°C prior to use.

8.2.1.5 PCR analysis

Polymerase chain reactions (PCR) were performed in a total volume of 25 µl, containing 15.8 µl MilliQ water (Millipore Corp, Bedford), 2.5 µl of 25 mM $MgCl_2$, 2.5 µl 10X PCR Gold buffer (Applied Biosystems, Foster City, CA, USA), 1 µl of 10 mM dNTP (Promega, Madison, WI, USA), 1 µl 10 µM of gene specific primer or control primer (β-actin) (Table 8.2), 0.2 µl of (1.5 U) Taq polymerase (Applied Biosystems) and 1 µl of each cDNA template. Amplification was performed in a Mastercycler epGradient S thermal cycler (Eppendorf, Hamburg, Germany) using the following thermal profile: enzyme activation for 10 min at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 54°C and primer extension for 90 s at 72°C, and final primer extension for 7 min at 72°C.

Table 8.2. Forward and reverse PCR primer sequences for the β-actin gene of Atlantic salmon (*Salmo salar*).

Primer	Orientation	Sequence
β-actin	FW	5'-ATG GAA GGT GAA ATC GCC-3'
	RV	5'-TGC CAG ATC TTC TCC ATG-3'

The amplification of each product was confirmed by 1% TAE gel electrophoresis. Gels containing 1 x 10^{-4} µg/ml ethidium bromide were electrophoresed at a constant 100V for 35 min in 1x TAE buffer. Gels were visualized under UV transillumination and photographed with a digital camera (Nikon, Coolpix). Product size was estimated using a 2-log DNA ladder (New Englands BioLabs, Ipswich, MA, USA).

8.2.2 Analysis of the antibody response to plasmid DNA encoded Neoparamoeba spp. peptides

8.2.2.1 Collection of sera from vaccinated fish

The fish used for assessment of antibody response came from the first targeted antigen vaccine trial described in Chapter 7. Prior to transport to the University of Tasmania a random sub sample of 20 fish, 10 from either pbS-Sfi (negative control) or 10 from each of the pbS-Sfi-7 clones (treatment) were selected and transported to CSIRO and maintained as outlined in section 8.2.1.2. All of the 20 fish were euthanised at 8 wk post-

vaccination (PV). Blood samples were withdrawn from the caudal vein using 21G needle and 3 ml syringe. Blood was allowed to clot overnight at 4°C and the serum was collected by centrifuging the blood samples at 1500 x *g*. All of the sera were then frozen and stored at -80°C until required.

8.2.2.2 Western blot analysis of antibody response

To examine the fish antibody response, a mixture of approximately 1 µg of each recombinant *Neoparamoeba* spp. protein (see Chapter 7) in reducing buffer (5 µl 4x NuPAGE LDS sample buffer; 2 µl 10x NuPAGE reducing agent) was reduced by heating at 70 °C for 10 min and 20 µl loaded on a NuPAGE Novex Bis-Tris 4-12% gel (Invitrogen). The proteins were separated by using constant 200V for 35 min and electrotransferred onto Hybond-P PVDF membrane (Amersham Biosciences, Foster City, CA, USA) in transfer buffer (25 mM tris, 192 mM glycine, 20% methanol) using a constant 100 mA for 90 min with cooling. Western blotting was performed using the WesternBreeze chromogenic immunodetection kit (Invitrogen) following the manufacturer's protocol. All antibodies used were diluted in blocking solution (Invitrogen). Random pools (3 fish per pool) of fish serum were applied at 1:50 dilution, mouse anti-salmon IgM was applied at 1:100 dilution and alkaline phosphatase-conjugated goat anti-mouse IgG was applied at 1:1000 dilution. After blocking and between each antibody incubation membranes were washed 3 times with TBS-Tween. The membranes were developed using BCIP/NBT (Invitrogen) and images were taken using a ChemiDoc™XRS, PC gel documentation system (BioRad, Hercules, CA, USA).

8.2.2.3 ELISA analysis of the magnitude of the response to SC10

Recombinant protein corresponding to clone SC10 was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 30 mM NaN_3; pH 9.6) (750 ng/well) and coated onto a Nunc-Immuno 96 well flat-bottom plate, MaxiSorp (Sigma, St. Louis, MO, CA, USA) and incubated overnight at 4 °C. After blocking in blocking solution (Invitrogen) for 1 h, fish sera (diluted 1:100 in blocking solution) were applied in duplicate wells for 1 h incubation followed by mouse anti-salmon IgM (diluted 1:100 in blocking solution) and alkaline phosphatase-conjugated goat anti-mouse IgG (diluted 1:1000 in blocking solution) each applied for 1 h incubation. Then, 100 µl/well alkaline phosphatase yellow

(pNPP) liquid substrate (Sigma, St. Louis, MO, USA) was applied for colour development. In between each of these steps, the wells were washed 5x with 300 µl/well of washing buffer (Invitrogen). After adding 25 µl/well of 3 M NaOH to terminate colour development, absorbances were measured at 405 nm using a DU 530 Life Science UV/Vis spectrophotometer (Beckman).

8.3 Results

8.3.1 Confirmation of foreign gene expression

8.3.1.1 pbS-Sfi and pDEST26 based plasmids drive foreign gene expression in Atlantic salmon muscle tissue

To confirm the ability of pbS-Sfi to drive *in vivo* expression of foreign genes in Atlantic salmon, samples from fish that were injected either with a positive control plasmid (pDEST26-S3A4) or with pbS-Sfi-S3A4 were analysed by RT-PCR. Both plasmids were able to drive expression of S3A4 *in vivo* (Fig 8.1a). The results indicate a true expression pattern as expression was neither detected in the same samples without reverse transcription (-ve RT) nor in non-injected site positive RT samples. The expression of β-actin in both injected and non-injected sites positive RT but not in negative RT samples confirmed RNA integrity and that expression of S3A4 was indeed a real observation and not due to background plasmid DNA carryover/contamination (Fig. 8.1b).

8.3.1.2 pbS-Sfi is able to drive expression of all six *Neoparamoeba* spp. genes in Atlantic salmon

After pbS-Sfi was confirmed to be a functional delivery vector, the *in vivo* expression of each of the six *Neoparamoeba* spp genes was confirmed when each plasmid was delivered individually. For the six fish that were separately injected with the six individual *Neoparamoeba* spp genes in pbS-Sfi vector, mRNA expression of that particular gene was detected in the muscle tissue surrounding the injection site (Fig. 8.2). Conversely no expression was detected in either the non RT controls (data not shown) or in non-injected muscle tissue. However, β-actin expression was confirmed in both injected and non-injected muscle tissue.

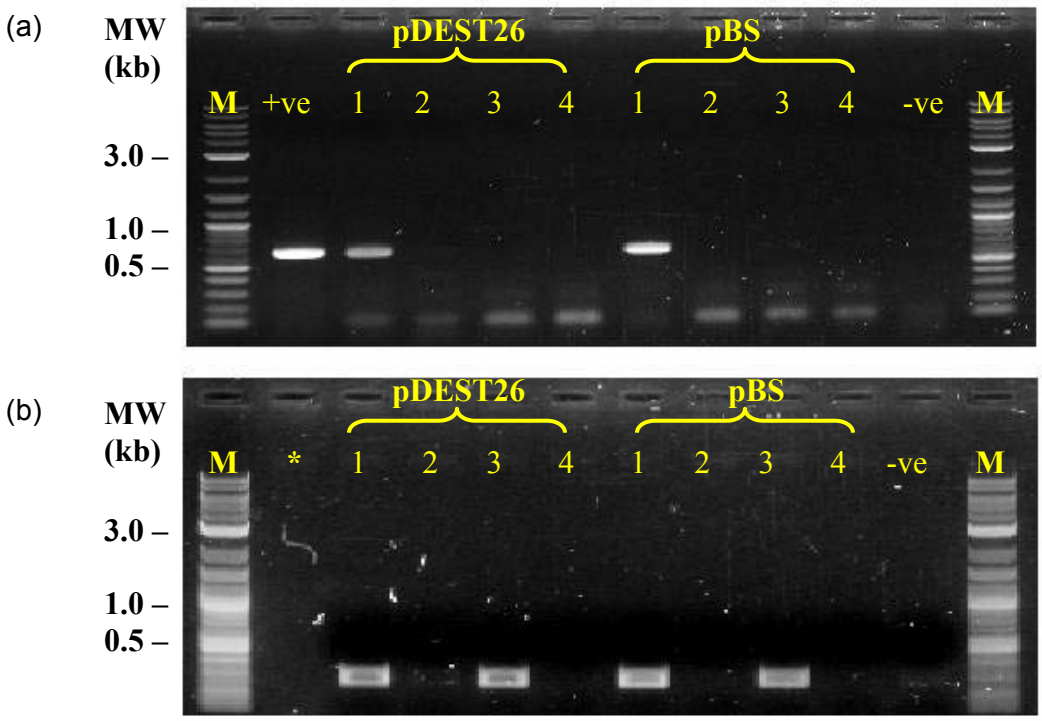

Figure 8.1. RT-PCR confirmation of plasmid driven S3A4 mRNA expression in Atlantic salmon muscle tissue (a) RT-PCR of S3A4 in samples from fish injected with either pDEST26-S3A4 or pbS-Sfi-S3A4 (b) Amplification of β-actin DNA by RT-PCR from fish injected with either pDEST26-S3A4 or pbS-Sfi-S3A4. (M) Marker (2-log DNA ladder; New England BioLabs), (+ve) positive control using pbS-Sfi-S3A4 plasmid as the template, (-ve) negative control using MilliQ water as the template, (1) injection site muscle tissue positive RT, (2) injection site muscle tissues negative RT , (3) non-injected site muscle tissues positive RT and (4) non-injected site muscle tissue negative RT, (*) no positive control was used for β-actin.

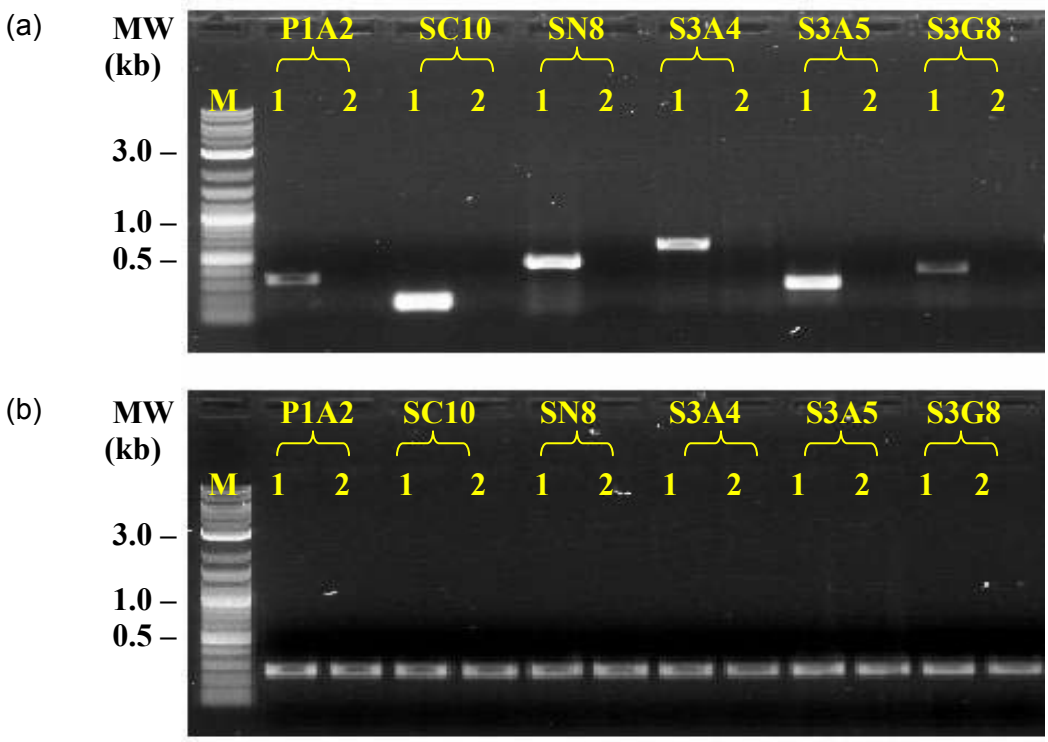

Figure 8.2. Confirmation of *in vivo* mRNA expression of all six DNA antigens when delivered individually to Atlantic salmon. Fish were injected with each antigen individually, muscle tissue collected 1 wk pi, cDNA prepared and subjected to PCR using (a) gene specific primers or (b) primers for β-actin. (M) Marker (2-log DNA ladder; New Englands BioLabs), (1) injected site muscle tissue positive RT-PCR, (2) non-injected site muscle tissues positive RT-PCR.

8.3.1.3 All six *Neoparamoeba* spp. genes are expressed when delivered simultaneously

To evaluate the expression of all six *Neoparamoeba* spp. genes when delivered simultaneously, muscle tissue samples from a single fish injected with an equal volume mix of pbS-Sfi plasmid DNA containing all six DNA antigens was assessed. All six genes were expressed when delivered simultaneously (Fig. 8.3). In general, the band intensity for SC10, S3A4 and S3A5 were strongest. Again, as a control, expression of mRNA was detected for β-actin in reverse transcribed RNA but not in non-RT RNA confirming expression of the gene and not just detection of the delivered plasmid DNA.

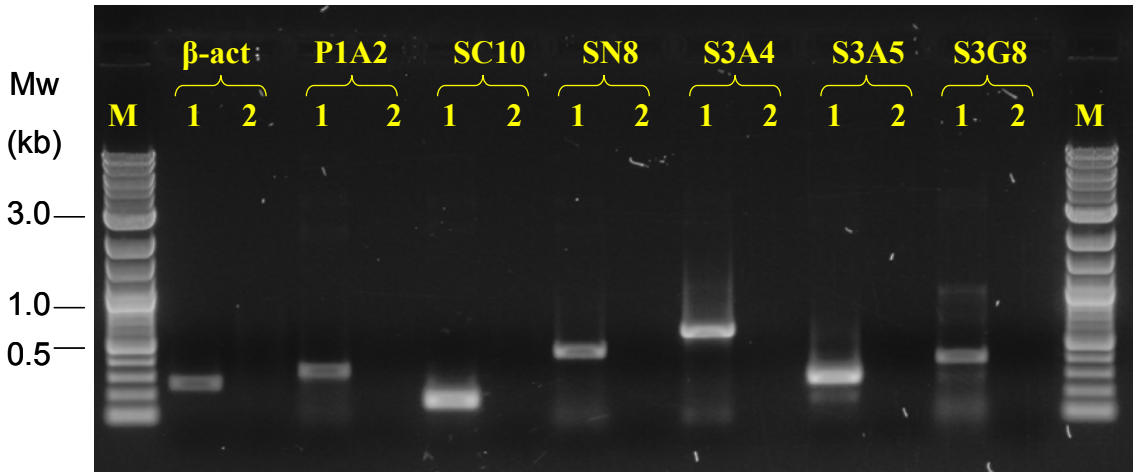

Figure 8.3. Confirmation of *in vivo* mRNA expression, at 1 wk p.v., of all six *Neoparamoeba* spp. genes when delivered simultaneously. (M) Marker (2-log DNA ladder; New Englands BioLabs), (1) injected site muscle tissue and positive RT-PCR, (2) injected site muscle tissues with negative RT-PCR

8.3.2 Analysis of the humoral immune response following DNA vaccination with six *Neoparamoeba* spp genes

8.3.2.1 Western Blot analysis to all 6 antigens

The antibody response of fish was assessed by screening fish serum against the antigens using Western blot. Results of the sera screenings showed that no binding with a pool of sera from negative control fish occurred (Fig. 8.4). Specific bands were detected using pooled serum (three fish/pool) from fish which were previously injected with an equal volume mix of plasmid DNA of each of the six genes. The size of the band detected was approximately 7-8 kDa and approximately corresponds to the predicted size of the SC10 antigen. No positive reaction was observed with either P1A2, SN8, S3A4, S3A5 or S3G8 (expected sizes 15, 24, 28, 15 and 24 kDa respectively).

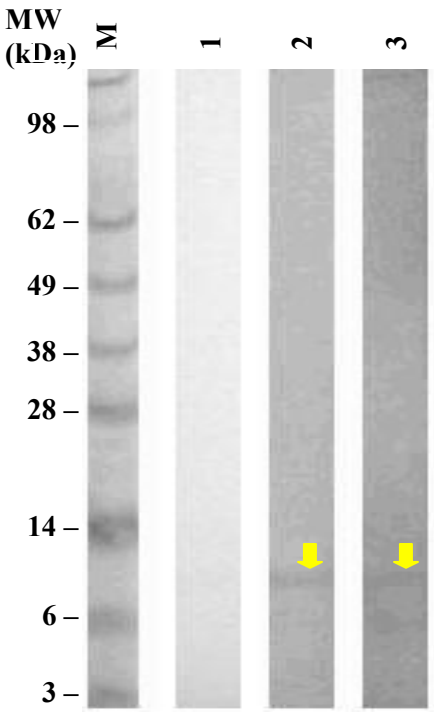

Figure 8.4. Western blot screening of fish injected with an equal mix of each of the six *Neoparamoeba* spp. antigens. Approximately 1 µg of each of the six recombinant proteins was reduced and run on a 4-12% NuPAGE Novex Bis-Tris gel. Proteins were transferred to Hybond-P PVDF. Proteins were probed with a pool of sera from three fish (diluted 1:50). (M) Marker, (Lane 1) a pool (10 fish) of serum from negative control fish which were only injected with naked pbS-Sfi, (Lane 2 and 3) pooled (three fish each) serum of fish which have been previously intramuscularly injected with an equal volume mix of each clone of interest in pbS-Sfi vector. Arrows indicate detected bands.

8.3.2.2 Magnitude of the response to SC10 antigen

Following detection of the SC10 antigen by pools of sera from injected fish the response of nine individual vaccinated fish was determined by ELISA. In general, each of the positive fish sera had significantly higher mean absorbance readings compared to the mean absorbance reading of negative fish serum (Fig. 8.5). The absorbance for individual sera ranged from 0.03 ± 0.002 (Fish #6) to 0.15 ± 0.03 (Fish #29) (mean ± S.E.). Apart from fish serum #23 and #29, the mean absorbances for all fish sera were lower than 0.08 but significantly higher than the negative control sera at the 0.05 level.

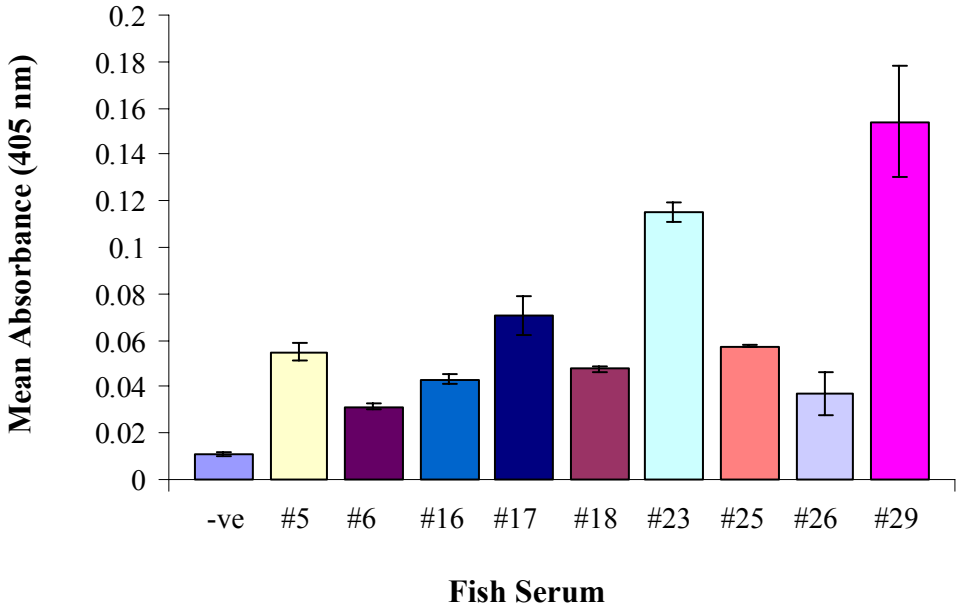

Figure 8.5. Mean absorbance (mean ± S.E.) of sera from nine DNA vaccinated Atlantic salmon. An ELISA plate was coated with 750 ng/well recombinant SC10 overnight at 4 °C, blocked with blocking solution (Invitrogen) and screened with each fish sera diluted at 1:100. Following incubation with mouse-anti-salmon IgA 100 µl PNPP liquid substrate was added. The reaction was stopped with 3 M NaOH (25 µl/well) and absorbance read at 405 nm using a DU 530 Life Science UV/Vis spectrophotometer (Beckman). (-ve) a pool of fish serum from three negative control fish which were injected with naked pbS-Sfi vector.

8.4 Discussion

Here we have shown *in vivo* mRNA expression at the site of injection of all six specific wild-type *Neoparamoeba* spp. genes when delivered in the pbS-Sfi vector, and also for S3A4 when in the pDEST26 vector. Expression in pbS-Sfi was confirmed either when the genes were delivered individually or simultaneously in a mixture. Furthermore, we have evidence that at least one antigen, SC10, elicits a significant humoral (antibody) immune response in vaccinated salmon. This provides some evidence that the increased RPS observed in the challenge trials reported in Chapter 7 is due to expression of the *Neoparamoeba* spp genes at the site of injection and the corresponding immune response.

The expression of all six *Neoparamoeba* spp genes in Atlantic salmon muscle at 1 wk pi is in accordance with other studies. For instance plasmid driven luciferase reporter gene expression *in vivo* in *Xiphophorus* sp. and rainbow trout, *O. mykiss* can be detected as early as 24 h pi and duration of expression is more than 10 and 115 days pi respectively for each species (Anderson et al., 1996) (Schulte et al., 1998). Moreover, *in vivo* transcription of the glycoprotein (G-protein) of viral hemorrhagic septicemia virus (VHSV) was detected in rainbow trout muscle at the site of injection at 7, 14 or 21 days after immunization (Boudinot et al., 1998). Similarly, expression of plasmid encoded VHSV G-protein or VHSV N-protein has been detected in the myocytes at the site of injected rainbow trout between 3-38 and 3-48 days respectively (Lorenzen and LaPatra, 2005). Previous studies have indicated that peak activity of foreign gene expression occurs within a wide range from 2 to 60 days pi (Gomez-Chiarri and Chiaverini, 1999; Rahman and Maclean, 1992). Although the level and duration of gene expression was not assessed in this study, *in vivo* expression for all genes was confirmed and it appears that the first step in eliciting an immune response to DNA vaccination against AGD, the expression of the delivered genes, does occur.

Differences in PCR band intensity indicates that some of the delivered genes may have a higher expression level than others. In the case of individually delivered plasmids, tissue samples were collected separately from different fish and differences in expression could be associated with differences in the amount of tissue sample used for RNA extraction and hence different quantities of starting RNA. The samples were subjected to crude PCR analysis and not quantitative analysis. Therefore, the amount of template RNA used in each PCR reaction was not the same. For simultaneously delivered plasmids, as only one muscle sample was taken to assess expression of all six genes together, total RNA template differences are unlikely to have affected expression levels. It is of course possible that a different copy number of each individual plasmid is being delivered for each gene. Although the same concentration (mass) of plasmid DNA was applied for each gene, as the size of each gene is different and hence the size of the plasmid different they would not be delivered in equi-molar ratios. Thus the actual copy number per μg DNA delivered would be different (Seelan et al., 2006), suggesting erroneous conclusions

relating to expression differences. It is perhaps possible to test the differences in expression levels, by delivering each of the six genes in an equi-molar ratio followed by quantitative real time-PCR (qRT-PCR) assay to determine the relative copy numbers of each of the antigens.

The logical second step in examining the response to DNA vaccination would be to determine whether the mRNA expression results in translation and subsequent protein expression. In rainbow trout, *in vivo* protein expression of plasmid delivered IHNV G-protein was determined with an immunoenzymatic test using Rabbit-anti-G-protein antibodies (Boudinot et al., 1998; Lorenzen et al., 1998). Likewise plasmid delivered *Vibrio anguillarum* OMP38 protein expression can be detected with a polyclonal antibody in Asian sea bass (*Lates calcarifer*) (Kumar et al., 2007). Fusion of the delivered gene to a reporter such as green fluorescent protein (GFP) has been used to demonstrate foreign gene expression following DNA vaccination of zebrafish (*Danio rerio*) with IPNV segment A (Mikalsen et al., 2004). Direct measurement of protein translation of the injected *Neoparamoeba* genes was not possible as we didn't have the antibody reagents available to measure this, nor were we able to attempt fusion to a reporter such as GFP. Generating these reagents was beyond the scope of this work. An indirect method to measure translation is to determine the antibody response to each of the six DNA antigens. This is reliant on actual translation occurring. But if a positive antibody response occurs translation can be implied. If there is no antibody response then translation would need to be measured directly using either the immunological methods used previously or fusion to a reporter such as GFP.

Antisera screening using both Western blot and ELISA demonstrated a positive antibody response to at least one of the antigens, SC10. From Western blotting, two pools of serum (3 fish per pool) from fish that were simultaneously injected with all six genes were found to contain SC10-specific antibodies when screened against a mix of all six recombinant proteins. Antisera screening of nine individual fish against SC10 recombinant protein alone further indicated the positive detection of the antibody response as all of the positive fish had a significantly higher mean absorbance (at a 1:100 sera dilution) than the negative fish. This antibody response is probably an indication of

an immune response triggered by the injection of plasmid DNA, given that *in vivo* gene expression had been confirmed and therefore translation to protein has most probably occurred.

The results from the western blot indicate that antibodies were produced only against one of the six recombinant proteins. Examination of antibody production following DNA vaccination in fish has shown that there is a wide discrepancy in terms of the number of responders and the magnitude of the antibody titre. For instance immunization of rainbow trout with plasmid DNA encoding either the IHNV-G protein or IHNV-N protein elicits a strong and a non-detectable antibody response respectively (Anderson et al., 1996). Although antibody responses to DNA vaccination were measured in spotted sand bass (*Paralabrax maculatofasciatus*) with OMP38 and 48 from *Aeromonas veronii* the titre used for detection was 1:10 (Vazquez-Juarez et al., 2005). The biological significance of a 1:10 dilution is questionable. Similarly a detectable response to *Vibrio anguillarum* OMP38 DNA vaccination has been reported for Asian seabass at a titre of 1:25 (Kumar et al., 2007). In our work we screened pools (3 fish ea) of sera at 1:50 which effectively meant that each fish sera were diluted 1:150. The results of the ELISA for SC10 suggest that responders have variable antibody levels. Therefore, it may have been that by combining sera (and therefore possible non-responding individuals) the resultant dilution used in the Western blot was too high for the detection of the remaining five recombinant proteins.

Another possible explanation for the lack of antibody response to genes other than SC10 could be differences in immunogenicity. When multiple genes are delivered via DNA vaccination either in a mixture or individually, different results regarding the stimulated immune response have been reported. In developing a DNA vaccine using pre-membrane and envelope genes for four serotypes of dengue viruses, effective antibody responses were stimulated to each gene and no interference among these different components was reported (Konishi et al., 2006). On the other hand, assessment of the immunogenicity of GMP-produced plasmids encoding five different *Plasmodium falciparum* proteins indicated a decreased T-cell response and antibody titre to each individual *P. falciparum* protein when they were delivered in a mixture compared to individual delivery (Sedegah

et al., 2004). Also, a study identified both a possible interference and synergy between plasmids encoding three separate HIV-1 regulatory genes when injected simultaneously (Kjerrstrom et al., 2001). As a result, it may be possible that antibody responses of some of the genes in our study have been affected by antagonistic interactions between two or more of the six antigens and subsequently reduced to a level which is insufficient for detection.

Antibody response following DNA vaccination is dependent on the nature of the gene delivered and the promoter which controls expression. DNA vaccination of goldfish with four different combinations of the SVCV-G gene containing full length and truncated forms of the G-gene, under the control of different promoters or fused to reporter genes had varying antibody responses (Kanellos et al., 2006). The majority of fish (4/6) injected with the full length and truncated forms under the control of the CMV promoter responded and demonstrated a high ELISA titre, as did fish injected with the fused reporter gene versions. However, only 1/6 fish injected with G-protein expression driven by different promoters such as SV40 or the muscle specific promoter MyHC had a measurable ELISA antibody response. This response was also on the threshold of detection. T cell responses, measured via a T cell proliferation assay also showed that the CMV driven and reporter gene fusion constructs had a small stimulation index (SI 1.55 and 1.8 respectively) whereas the group injected with the other promoters had a stimulation index almost double (SI 3). Like the antibody titres the number of responders also differed between these groups. However based on limited information, it appears that the promoter used in our study, the Atlantic salmon β-actin promoter, did not contribute to the differences (or lack thereof in case of five antigens) in immune responses, as the promoter successfully expressed all the six antigens in the muscle tissue including that of SC10, for which an antibody response was discernable. Thus it appears that the expressed antigens themselves may have influenced the type of immune response. It is likely that the remaining five antigens themselves could have skewed toward a cellular response and not a humoral (antibody) response, and hence led to the failure to detect the corresponding recombinant proteins on a Western blot, unlike SC10.

Our failure to detect the five recombinant proteins could also be due to technical reasons associated with the form of the antigens or the blotting procedure itself. For instance, all proteins were recombinantly produced in a bacterial system, whereas protein production in fish muscle would occur in the native eukaryotic cells. Proteins produced in eukaryotic systems undergo a number of post translation modifications that can affect structure. This in turn affects the nature and exposure of antigenic determinants. SC10 is a very small protein (approx 6-7 kDa), and it is possible that due to its size there are only small differences between the bacterial recombinant and eukaryotic recombinant forms. Therefore antibodies produced could recognize the bacterial recombinant form. Also the state of the proteins could affect binding. All six proteins were reduced prior to running on the SDS-PAGE gel. In some instances, fish Ig does not bind denatured protein (Arkoosh and Kaattari, 1991). Finally, all of the recombinant proteins were co-loaded and screened with pooled fish serum during Western blot anti-sera screening. It may be possible to increase the sensitivity by further screening sera from individual fish (unpooled), by producing the recombinant proteins in a eukaryotic system, screening each recombinant protein separately and running the native and denatured versions of each protein.

It should be noted that failure to detect an antibody response to the delivered *Neoparamoeba* genes does not necessarily imply that no immune response was stimulated by these genes. It is known that when DNA immunization is used, the cellular immune response is important in providing protection. For instance, following plasmid immunization in rainbow trout with pCMV-vhsV there was no detectable antibody titre in blood samples taken at 67 days pi (Lorenzen et al., 1998) despite an RPS of 98%. Furthermore, blood taken 40 days pi from the survivors of a VHSV challenge had no detectable antibody titre despite a RPS of 66%. These results were similar for DNA vaccination using the IHNV-G protein whereby no neutralising antibody titre was measurable in injected rainbow trout despite an RPS of 100% (Corbeil et al., 2000). This suggests that some other immunological mechanism may be responsible for protection such as cellular immunity, although this was not ascertained. In mammals MHC class I-restricted CTL and Th1-type immunity (Tighe et al., 1998) are important in providing

protection following DNA immunisation. For example, following DNA vaccination against the protozoan parasite disease, Leishmaniasis, a predominant Th1 immune response was reported to provide effective protection against the disease despite a negligible antibody response (Coler et al., 2002). Cell-mediated immunity is continually being shown to play an important role in the protection against many pathogens (Nakanishi et al., 1999). In common with most *Herpesviridae*, the protection against re-infection by spring viraemia of carp virus (SVCV) is associated with cell-mediated immunity and not with virus neutralising antibodies (Dixon, 1997). Therefore, it is entirely possible that the vaccination against AGD with the six different *Neoparamoeba* spp. genes predominantly drives a cellular immune response and that this may be responsible for subsequent protection. However, this is only speculation and definitely warrants further investigation.

8.5 Summary

In summary we have shown that injection of Atlantic salmon with a six antigen DNA vaccine coding for *Neoparamoeba* genes results in mRNA expression of these foreign genes in injected muscle tissue. At this stage we are uncertain whether this mRNA expression results in protein translation of all six antigens. However, we have indirect evidence that this occurs for at least one of the antigens, SC10, as we can detect a measurable antibody response to recombinant SC10 in both Western blot and ELISA. Failure to detect the other five antigens could be due to lack of protein translation or to the nature of the response to the DNA vaccination, the form of recombinant protein or the sensitivity of Western blot procedure employed. Further work is warranted and should include production of recombinant proteins in a eukaryotic system and production of antibodies to each of the six proteins for use in protein expression assay procedures. Finally assessment of the actual nature of the response (humoral vs cellular immunity) is also warranted.

9. BENEFITS AND ADOPTION

The Tasmanian Atlantic salmon industry is the principal beneficiary of the research undertaken during this project. This arises through the provision of:

- A crude six antigen DNA vaccine that increases survival from AGD in a laboratory challenge system
- Data showing that when co-administered all six antigens are expressed as mRNA in skeletal muscle of Atlantic salmon
- Data showing that one antigen, putatively named SC10, is capable of eliciting a humoral immune response in injected Atlantic salmon

The project outputs have been adopted through;

- New funding via FRDC, Aquafin and Seafood CRCs and CSIRO for phase III of the vaccine development
- Full support from TSGA and Huon Aquaculture Pty Ltd on behalf of the industry for sea trials of the crude vaccine

The project also contributed to the education programme of the Finfish CRC through the successful completion of a Masters degree by Ms. Lim.

10. FURTHER DEVELOPMENT

The research reported herein demonstrates the possible feasibility of a DNA vaccine for AGD. However, the challenges of translating the encouraging results from the laboratory to the field and commercialisation remain. This will be the focus of FRDC project 2007/234 ‘AGD Vaccine Phase III; Sea-based trials, refinement and commercialisation’ which commenced in July 2007. The focus of this next phase is to conduct sea trials with a real-life chronic infection and to conduct more research on:

- Vaccine refinement including optimisation of the delivery vector, the dose and timing of vaccination.
- Further research on the feasibility of protein vaccination.
- Confirmation of the mechanism of response and protection afforded by the vaccine.

11. PLANNED OUTCOMES

The expected outcome of this project was protection of Atlantic salmon to AGD by DNA vaccination. This project has provided strong evidence that a six antigen DNA vaccine is able to significantly increase survival in fish challenged with AGD within the University of Tasmania's challenge system. Furthermore, there is anecdotal evidence that the vaccine is able to significantly reduce gill scores of challenged fish. The outcome from this project will now be a sea trial of the crude six antigen vaccine and further laboratory based challenges in order to refine the vaccine.

In the Aquafin CRC Commonwealth Agreement, two outcomes were stated in relation to AGD vaccine development.

The first, "Clear evidence of feasibility of native or subunit vaccine" has been achieved by this project. The project has also made major strides towards the second, "Provide the capability for a commercial partner to develop a vaccine providing substantial prevention of mortality resulting from AGD infection".

12. CONCLUSIONS

This project has successfully achieved its objectives.

1. *To identify potential protective antigens from N. pemaquidensis using a combined DNA/protein approach.*

Using the approach of Expression Library Immunisation (ELI) we rationalized a very crude expression library consisting of 1100 clones, down to a potential protective antigenic fraction of 288 clones. Further, through the power of sequencing and bioinformatics we were able to achieve deconvolution of the library and identified 39 unique contributing clones of which five warranted further investigation. These combined with the bio-informatic analysis of cDNA's cloned into a subtractive library facilitated the identification and construction of an experimental six antigen DNA vaccine against AGD.

2. *To demonstrate protection of Atlantic salmon against clinical amoebic gill disease via cDNA and/or recombinant protein vaccination.*

Through vaccination/acute AGD challenge experiments we were able to demonstrate that the six antigen DNA vaccine provides a relative increase in protection of approximately 40%. We also provide evidence that the vaccine can reduce the percentage of AGD affected gill filaments in surviving fish following a more chronic infection. Results of a limited trial suggest that bacterially derived recombinant proteins do not increase protection to AGD. However, further investigation into protein vaccination may be warranted.

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APPENDIX 1: Intellectual Property

The six clone vaccine referred to herein is subject to a provisional patent (#2007903901) filed for an on behalf of the Aquafin CRC and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and complete specifications filed on 1st October 2007, in Australia as Application No. 2007221759, in USA as Application No. 11/906,440, and in Canada – Application No. 2605130.

APPENDIX 2: Staff

Staff engaged directly on this project:

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