Final Report

Aquafin CRC

Atlantic salmon Aquaculture Subprogram:
Development of an AGD vaccine: phase II (UTS)

Robert Raison, Margarita Villavedra, Kevin Broady, Michael Wallach, Joyce To, Susan Lemke, Rohan Panwar, Gaganpreet Sandhu, Philip Crosbie, Michael Attard, Mark Adams, Mark Powell and Barbara Nowak.

June 2008

Aquafin CRC Project No: 3.4.4(2)
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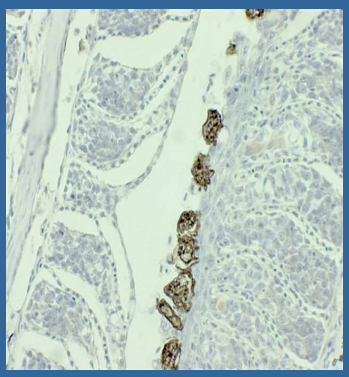

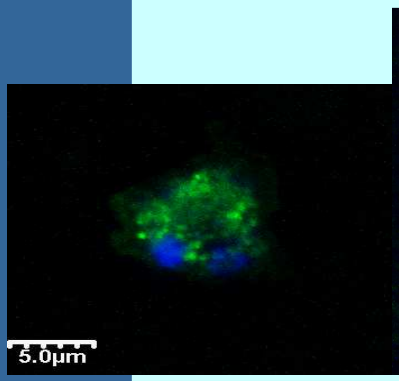

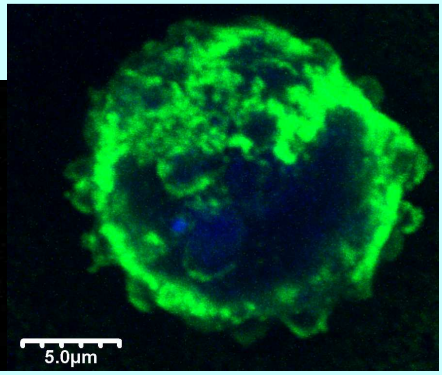

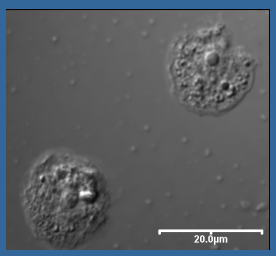

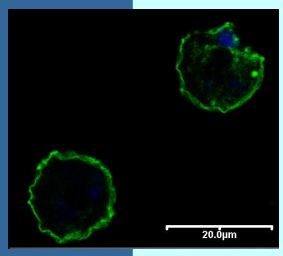

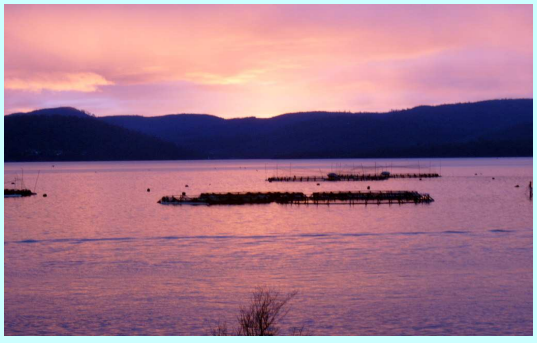

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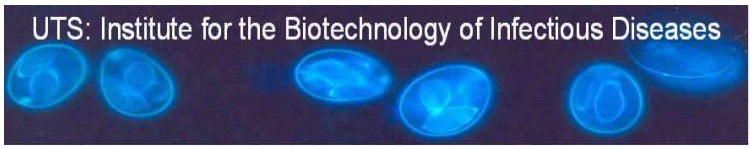

Tasmanian Aquaculture & Fisheries Institute
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Table of Contents

NON-TECHNICAL SUMMARY

CRC: 3.4.4(2)/FRDC: 2004/217.2
Atlantic Salmon Aquaculture Subprogram: Development of a vaccine for amoebic gill disease: Phase II (UTS)

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OBJECTIVES:

1. To identify potential protective antigens from *Neoparamoeba* spp using a combined DNA (CSIRO)/protein (UTS) approach.

2. To identify and characterize attachment molecules involved in the infection process of Atlantic salmon by *Neoparamoeba* spp.

3. To demonstrate protection of Atlantic salmon against clinical AGD via cDNA and/or recombinant protein vaccination.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE:

This project has increased our knowledge of the complex array of glycans and glycoproteins expressed on the surface of infective *Neoparamoeba*. Cell surface antigens associated with attachment of the parasite to host tissue and unique to infectious *Neoparamoeba* have been identified using monoclonal antibodies. Of particular importance is the discovery of an “immunosuppressive” component present within the high molecular weight glycoproteins from infective *Neoparamoeba*. This finding, together with an indication of a protective effect derived from the use of oil emulsion adjuvant alone, forms the basis of further experiments aimed at optimising vaccination strategies for AGD.

- A panel of anti-*Neoparamoeba* monoclonal antibodies (MAbs) has been developed. Fifty of these MAbs are specific for infective parasites and they reveal that the majority of the antigens uniquely expressed on infective parasites are carbohydrate in nature. This panel of antibodies is available as a resource for further AGD research.

- Several MAbs were shown to have the capacity to block the binding of infective parasites to salmon gill tissue. This group of MAbs recognise the same cluster of glycoproteins expressed on the surface of infective parasites. These glycoproteins are contained in a high molecular weight preparation from infective parasites. While non-infective parasites contain a similar set of high molecular weight glycoproteins they are not recognised by the infective parasite-specific MAbs. This high molecular weight antigen (HMWA) fraction from infective *Neoparamoeba* constituted a potential target for vaccine development.

- A vaccination trial with HMWA from infective parasites revealed that this fraction contains unidentified components that can down regulate the protective immune response in salmon. This finding provides the basis for further detailed analysis of the immunomodulatory factor or factors with a view to optimising the protective effect of any potential vaccine against AGD.

- An unexpected outcome from the HMWA vaccination trial was the finding that injection of adjuvant alone resulted in a significant level of protection from AGD. This suggests that innate immune mechanisms may play an important role in protecting susceptible fish from infection with *Neoparamoeba*.

KEYWORDS: Amoebic gill disease, Atlantic salmon, *Neoparamoeba*, vaccine, attachment proteins.

ACKNOWLEDGEMENTS

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Finally, we wish to thank Dr Peter Montague (Aquafin CRC), Dr Patrick Hone (FRDC) and Pheroze Jungalwalla TSGA) for their continued support during the course of this project.

BACKGROUND

This project is one of six within the Aquafin CRC Health Program that comprise a portfolio of projects addressing the problem of amoebic gill disease **(AGD)** in the Australian Atlantic salmon industry. The project is the second phase of a program of research aimed at developing a vaccine for AGD that was initiated in the Aquafin CRC in 2002 as part of the Host–Pathogen Interactions in Amoebic Gill Disease Project (Aquafin CRC Project 3.4.2). Phase 1 of the project was completed in 2004.

AGD is considered to be the most significant health problem for farmed Atlantic salmon in Tasmania, costing the industry an estimated $15 million pa. 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 and more than one treatment is usually required. In addition, it is very labor-intensive, requires large volumes of freshwater, and is stressful to the fish, which further impacts on their health. 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. The essential objective is to develop a set of strategies and tools to provide the salmon industry with a substantial reduction of 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 fish pathogens, and considerable research has been conducted on vaccines for both bacterial and viral pathogens of fish. In a recent, novel approach to immunoprophylaxis in trout, Lorenzen *et al*. (2000) injected fish with the genes encoding a mouse recombinant single-chain antibody against viral hemorrhagic septicaemia virus and showed that the resulting circulating antibodies provided protection from challenge by the infective virus. Such an approach may be more broadly applicable to systemic viral infections where circulating antibodies offer a significant level of protection. However, the administration of foreign genes to aquaculture species may raise both regulatory and industry concerns. Despite these interesting developments in immunoprophylaxis for fish diseases, relatively little work has been done on vaccines against parasitic diseases of fish. One of the rare examples of progress in this area has been the development of an experimental recombinant vaccine against the ecto-parastic ciliate, *Ichthyophtirius multifiliis*, based on a surface immobilization antigen which induces a protective immune response in fish (He,1997).

Significant advances have occurred in the development of vaccines for parasites in humans and traditional domestic animals, using either recombinant or synthetic peptides. However, while experimental vaccines have shown promise for diseases such as malaria (Sedegah*,* 1994), Chagas disease (*Trypanosoma cruzi* infection; Costa*,* 1998) and cysticercosis (Manoutcharian, 1998) there are currently no approved vaccines for any of the multitude of human parasitic diseases.
In the area of domestic livestock, UTS researchers (Wallach, Smith and colleagues) in collaboration with ABIC Ltd., Israel, have developed a maternally based subunit vaccine against coccidiosis in chickens (CoxAbic®), which has been field tested, registered and marketed in Israel, South Africa, Thailand, Argentina and Brazil.

Experiments conducted prior to the establishment of the Aquafin CRC indicated that immunisation with crude, whole parasite preparations did not result in significant levels of protection from infection, despite the production of specific antibody in the

serum of treated fish. While studies in other parasitic diseases in other species have yielded similar negative results with crude antigen preparations, the use of subunit or single antigen preparations has resulted in the development of protective immunity in a number of well documented cases (Smith, 1994). Furthermore, studies on microsporidiosis caused by *Encephalitozoon cunucli* have demonstrated the feasibility of developing an anti-parasitic subunit vaccine, even under circumstances where natural infection does not lead to a protective immune response (Didier, 1999). A similar situation exists in AGD where recovery from infection has not been clearly demonstrated to protect from re-infection. To our knowledge, no commercial recombinant vaccine directed against protozoan diseases for humans, terrestrial animals or fish has been produced. Thus this project has been viewed as high-risk since its conception and the development of such a vaccine would be ground breaking.

Attachment of *Neoparamoeba* spp to healthy gill epithelium precedes the formation of epithelial lesions and ensuing disease (Zilberg, 2000). Following attachment, the amoebae induce hyperplasia of the lamellar epithelium of the gills, resulting in fusion of secondary lamellae and formation of macroscopic mucoid gill patches (Dykova, 1998; Clark, 1999; Adams, 2001). Thus, attachment of the parasite and the induction of host cell hyperplasia are key targets for vaccine-directed intervention with respect to this disease. The attachment of *Neoparamoeba* to the gill epithelium of salmon is likely to be mediated by specific receptor-ligand interactions between molecules expressed on the surface of parasite and host cells. The molecular basis for the attachment of a related parasite, *Entamoeba histolytica* to its target cell or tissue has been well documented. A galactose-binding lectin has been shown to play a key role in the attachment of *Entamoeba histolytica* to host cells (McCoy, 1994). It's known that *Entamoeba histolytica* first colonises the mucus layer by adherence via a parasite surface Gal-lectin to galactose and N-acetyl-D-galactosamine (GalNAc) present in the mucus (Moncada, 2003). Similarly, a glucose specific lectin of *Giardia lamblia* (Farthing, 1986) and a sialic-acid specific lectin from *Tritrichomonas mobiliensis* (Babal, 1999) have been characterised as mediators of attachment.

In order to characterise and isolate the molecules involved in the attachment and adherence of *Neoparamoeba* to the gill epithelium, we hypothesized that only wild type, infective parasites contain the molecules required for binding to the host cells, whereas the culture grown, non-infective *Neoparamoeba* lack these attachment factors rendering them harmless to fish. Thus, analysing differences at the molecular level between these two types of parasites would lead to the identification of the molecules involved in attachment, infectivity and pathogenesis. These molecules would in turn provide targets for vaccine development.

To identify *Neoparamoeba* surface molecules that mediate attachment to gill tissue we have been developing polyclonal and monoclonal antibodies (MAbs) against *Neoparamoeba* spp. The panel of MAb was screened by immunofluorescence and flow cytometry to identify those that recognize antigens expressed on the surface of the wild type (infective) parasites. Some of these monoclonal and polyclonal antibodies were assessed for their ability to block the binding of *Neoparamoeba* to gill epithelial cells in an attachment assay developed at TAFI, University of Tasmania. Antibodies that inhibit parasite attachment were then used to identify and characterise attachment-associated molecules.
In the initial phase of this project we produced polyclonal and monoclonal antibodies against *in vitro* cultured (non-infective) PAO27 parasites due to the lack of purified wild type parasites. Analysis with these antibody reagents has revealed clear differences in the antigen profiles expressed by wild-type infective parasites versus those of parasites that have been in culture for extended periods (Villavedra, 2005).

Subsequently, cell fusion experiments using a subtractive immunization method (Matthew, 1987; Brooks, 1993; Sleister, 2002) were performed with the objective of enhancing the potential for development of MAbs specific for antigens expressed on wild type (infective) parasites. Using this methodology we obtained a high percentage (89%) of MAbs specific to wild type parasites, but only a few specific for peptide epitopes with the required affinity suitable for testing in the attachment assay. In this second phase of the project, we produced more hybridomas using a similar methodology but pretreating parasites with glycosidases in order to deplete them of carbohydrate epitopes (Miguez, 1996) and thus enhance the likelihood of obtaining peptide-specific MAbs.

The vaccine strategy proposed in this project is based on prevention of attachment of the parasite to the gill epithelium of the host, and for this to occur an effective immune response must be induced at the mucosal surface of the gills. Previous studies performed at UTS demonstrated that a significant antibody response could be detected in mucus from trout immunised intraperitoneally and boosted by challenge via a mucosal surface (gut) (Cain, 2000). Based on this work, experiments were carried out in this project to investigate an effective immunization strategy for inducing *Neoparamoeba* specific antibodies in gill mucus.

During the course of this project studies at the University of Tasmania using 18SrRNA gene probes identified *Neoparamoeba perurans* as the aetiological agent of AGD in Tasmanian farmed Atlantic salmon (Young, 2007). *Neoparamoeba pemaquidensis*, previously thought to be associated with disease (Douglas-Helders, 2001), was not found to be associated with AGD lesions. This finding has no direct impact on the studies carried out in this project as the infective amoeba used throughout this project (referred to as *Neoparamoeba* spp) were isolated from the infection tank (D1) at University of Tasmania. These amoebas have been shown to be predominantly *N. perurans* (Young, 2007).

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 estimated to be approximately $15 million and represent a very 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 target the potential rewards justify the risk.

OBJECTIVES

4. To identify potential protective antigens from *Neoparamoeba* spp using a combined DNA (CSIRO)/protein (UTS) approach.

5. To identify and characterize attachment molecules involved in the infection process of Atlantic salmon by *Neoparamoeba* spp.

6. To demonstrate protection of Atlantic salmon against clinical AGD via cDNA and/or recombinant protein vaccination.

METHODS

1. Parasites

a) Cultured parasites
PA027 is a non-infectious clone of *Neoparamoeba pemaquidensis,* originally isolated from AGD affected salmon and established in continuous culture since 1994. These parasites were grown on malt yeast seawater agar plates as described in Villavedra *et al.* (2005).

b) Freshly isolated or wild type (WT) parasites
The freshly isolated (infective) *Neoparamoeba* spp. was obtained directly from the gills of individual salmon from the infection tank (D1) maintained at the Aquatic Centre of the University of Tasmania. The gills were excised from dead fish and amoebae isolated according to the method of Morrison *et al.* (2004).

2. Antigens

Parasites were centrifuged at 400 x g for 15 min at 10 ^{o}C, washed in PBS and treated as follows:

a) For ELISA – Washed parasites were resuspended in PBS-0.05% sodium azide (Sigma, USA) and Complete Protease Inhibitor Cocktail (Roche, Switzerland) according to the manufacturer's instructions and sonicated for 15 cycles of approximately 38 watts for 15 sec, with 45 sec intervals between cycles on ice using a Misonic 3000 sonicator. The suspension was then centrifuged at 20,000 x g for 15 min and the supernatant stored at -80^{o}C.

b) For immunoblot – Washed parasites or gill scrapings from non-infected fish were resuspended in SDS-PAGE sample buffer, heated in a boiling bath for 15 min, centrifuged at 20,000 x g for 15 min, and the supernatant stored at –80 ^{o}C.

c) For immunodepletion – Washed PAO27 parasites were resuspended in PBS and sonicated for 10 cycles of approximately 38 watts for 10 sec with 30 sec intervals between cycles on ice using a Misonic 3000 sonicator. The suspension was aliquoted and stored at -80^{o}C.

d) For positive immunisation – Four types of antigens were used for immunisation; untreated sonicated whole parasite antigen and sonicated membrane fraction either treated or untreated with Peptide- N-glycosidase F (PNGase F), O-glycosidases and neuraminidase. The membrane fraction was prepared by osmotic lysis of washed, freshly isolated parasites using PBS diluted 1:1 in distilled water. The parasite suspension was frozen at -20 ^{o}C, thawed at 37 ^{o}C and centrifuged at 100,000 x g for 1 hour. The pellet was directly sonicated (untreated) or treated with PNGase F (0.4 U/μg protein; Roche, Switzerland), O-glycosidase (0.5 mU/μg protein; Roche, Switzerland) and neuraminidase (0.4 mU/μg protein; Roche, Switzerland) for 4 h at 37 ^{o}C. Freshly isolated parasites and membrane preparations were submitted to 10 cycles of sonication as above, aliquoted and stored at -80 ^{o}C. Protein concentrations of all preparations were determined using the BCA Protein Assay Kit (Pierce).

e) Parasite soluble fraction - A suspension of approximately 6 x 10^{7} WT amoeba/mL was centrifuged at 16000 x g for 15 min at 10^{o}C. The pellet was then submitted to osmotic lysis by re-suspension in 250 μL of MilliQ water with Complete protease inhibitor cocktail (Roche) and sodium azide (Sigma). The parasite suspension was

frozen at -20 °C, thawed at 37 °C and centrifuged at 16000 x g for 15 min at 10°C. The pellet obtained was submitted to two further freeze/thaw cycles and the supernatants were pooled and stored at -80 °C. Protein concentration was determined using the BCA Protein Assay Kit (Pierce).

3. *Subtractive immunisation*

The general subtractive immunisation scheme used to elicit MAbs specific for infective parasites is outlined in Fig 1. For immunodepletion 6 to 8 week old Balb/c mice (Gore Hill Research Laboratories, Australia) were injected i.p with antigen (equivalent to 1 x 10^6 PAO27 parasites) followed 24 and 48 h later by an i.p. injection of 100 µL of a 20 mg/mL solution of cyclophosphamide monohydrate (ICN, USA) in PBS. This cycle was repeated once for fusion 8 or 3 times for fusions 2, 3, 6 and 7 with two week intervals between cycles. This treatment was followed 7 to 10 days later by positive immunisation, which involved priming with sonicated infective WT parasites emulsified in Freund's Incomplete Adjuvant (FIA) with the exception of fusion 8 where the priming antigen was WT membrane preparation emulsified in Monophosphoryl Lipid A and Trehalose Dicorynomycolate (MPL+TDM) Adjuvant System (Sigma, USA). Booster immunizations were performed as described in Table 1. Three days before the fusion, mice were boosted i.p. with the respective antigen preparation equivalent to 3 x 10^5 parasites (see Table 1). Two mice, to be used as immunisation control, were inoculated with antigen for immunodepletion following the protocols above but were not treated with cyclophosphamide (non-immunodepleted).

When immunodepletion was performed, the time between antigen boosts in the positive immunisation phase of the protocol was approximately 2 weeks because the window of immunodepletion normally last about 5 weeks after the last cyclophosphamide injection (Sleister, 2002). When immunodepletion was not used the time between antigen boosts was 3 to 5 weeks.

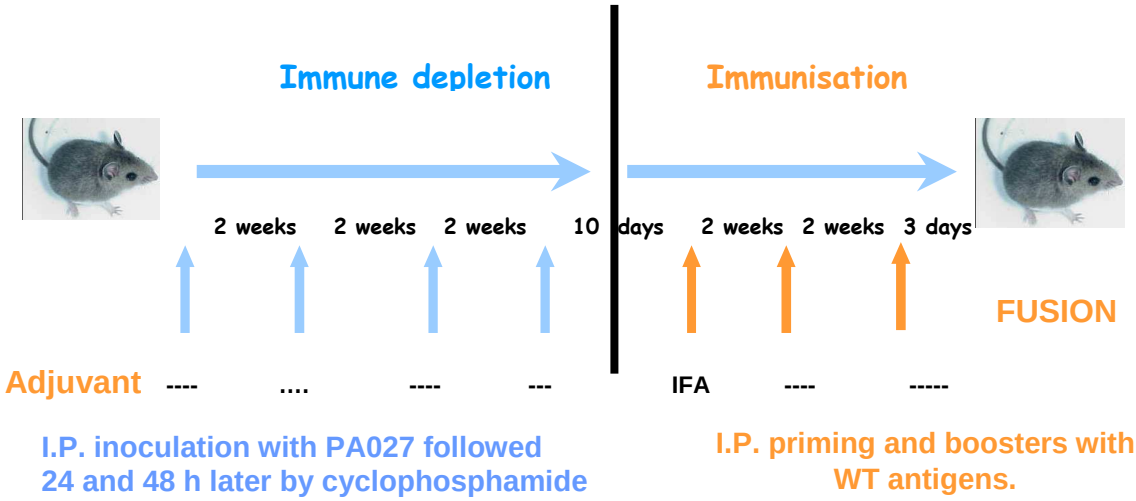


Figure 1: Subtractive immunization protocol

4. *Development of hybridomas*

Spleen cells from immunised mice were fused with NS-1 cells (CSL, Australia) by exposure to 50% polyethylene glycol (Ajax, Australia) according to standard protocols. Clones growing in HAT selective media (RPMI 1640, 100μM hypoxanthine, 0.4 μM aminopterin, 1.6 μM thymidine, 20% FCS, 50 μg/mL gentamicin) (Sigma, USA) were expanded and tested for antibody production. Hybridomas secreting specific antibodies were selected by ELISA screening using PAO27 and WT ELISA antigens. Hybridomas producing antibodies that bound to WT parasite antigen in ELISA, and that were non-reactive with the PAO27 antigen, were further tested by IFAT to identify antibodies that reacted with cell surface antigens. Hybridoma cultures producing antibodies against surface molecules unique to WT parasites were cloned by limiting dilution.

5. Production of polyclonal antibodies.

Strategy 1: Depletion of WT antigen by affinity chromatography using anti-PAO27 antibodies.

The crude immunoglobulin fraction of a rabbit anti PA027 antiserum produced by P. Crosbie (TAFI, University of Tasmania) was coupled to CNBr-Sepharose beads and the optimum ratio of anti-PA027 antibody:WT sonicated antigen determined for a one step batch depletion procedure. Different batches of WT sonicated antigen were depleted by absorption with 20% excess of the calculated optimum amount of immobilised antibody in order to assure complete depletion of the sample. WT antigen was passed over the anti-PA027 column and unbound material collected and pooled as the fraction enriched for WT-specific antigens (i.e. depleted of antigens recognised by anti-PA027). Antigens bound to the anti-PA027-Sepharose were sequentially eluted with glycine, pH2.5, NaSCN and Glycine/DMSO. The various antigen fractions arising from this procedure were analysed by ELISA and SDS-PAGE.

Strategy 2: Depletion of cross-reactive antibodies using PAO27 antigen adsorbed onto PVDF membrane.

A rabbit was immunised with whole sonicated WT (infective) parasites and antibodies cross-reacting with the non-infective PA027 parasites were removed by incubating the diluted rabbit serum with PA027 antigen adsorbed to PVDF membranes. The original antiserum and the PA027-adsorbed serum were analysed by ELISA against WT and PA027 antigens.

An additional rabbit was immunised with WT membrane preparation (see 2 d)

6. *In vitro attachment assay protocol*

Eighty thousand WT amoebas were re-suspended in 1 mL of the test antibody diluted in PBS or 1 mL of sterile sea water and incubated for 30 min at room temperature.

Two non-infected Atlantic salmon were sacrificed by overdose of anaesthetic, exsanguinated and perfused completely. The gills of the fish were excised and placed in transport medium (25μg/mL streptomycin sulphate, 25μg/mL penicillin, 25μg/mL carbenicillin, 62.5 μg/mL ampicillin and 25μg/mL erythromycin sterile seawater). The samples were kept on ice and maintained in transport medium for no more than 30 min. Each gill basket was rinsed under a gentle stream of filtered seawater and dissected into single gill arches and segments. Five to 15 filament segments were placed into a siliconised 12 wells tissue culture plate that was

previously filled with 1 mL of Tissue maintenance medium (L15 media supplemented with 0.01% FCS, 5mM L-glutamine, 1% v/v Pen/Strep solution and 0.05 mg/ml gentamicin and osmolality adjusted to 780 mOsmol with NaCl).

Ten thousand treated amoebas, in a total volume of 250 µL, were slowly added to each well directly onto the gill filament to maximise exposure and opportunity for adherence. The plate was incubated at 18ºC for 24 hours and each gill segment rinsed gently in a stream of seawater. The gills were scraped into a new 12 well tissue culture plate and allowed to adhere for 2 h. The supernatant was discarded, the plate washed twice with 1 mL of sterile seawater and the number of adherent amoeba per well counted in an inverted microscope.

Each assay consisted of 4 replicates per fish and 2 fish were used for each assay. Each MAb has been tested at least twice due to the variations within the assay. Statistical analysis of the results were performed using Kruskal-Wallis Non-Parametric Test and Dunn's Multiple Comparison Test using GraphPad Prism program version 4.03

7. *ELISA*

a). Hybridoma screening **-** Maxisorp ELISA plates (NUNC) were coated with 100 µl/well of a 5 µg/ml solution of ELISA antigen and blocked with PBS-1% BSA. After regular washes 100 µl/well of each MAb were dispensed and incubated for 3 h at 37^{o}C. Purified mouse IgG1 antibody (MOPC-21, Sigma, USA) and purified mouse IgMκ (MOPC-104E, Sigma USA) were used as isotype controls. Plates were then washed and incubated with alkaline phosphatase conjugated second antibody (anti-mouse IgG, γ-chain specific and/or anti– mouse IgM, µ - chain specific; Sigma, USA) for 90 min at 37^{o}C. The plates were finally washed and colour developed with 200 µl/well of p-nitrophenylphosphate solution (Fast Tablet sets, Sigma) for 1 h. The OD at 405 nm was measured at 30 minutes and 1 h.

b). Quantification of *Neoparamoeba* specific antibodies in salmon
Maxisorp ELISA plates (NUNC) were coated with 100 µl/well of a 5 µg/ml solution of sonicated *Neoparamoeba* antigen and blocked with PBS-1% BSA. After washing 100 µl/well of sample or standards (a pool of serum samples from the immunised groups), were dispensed and incubated for 3 h at 37^{o}C. Mouse anti-salmonid immunoglobulin MAb 5F12 alkaline phosphatase conjugated (Immuno-Precise) diluted 1/1000 was used as secondary antibody. The plates were washed and colour developed by addition of 200 µl/well of p-nitrophenylphosphate solution (Fast Tablet sets, Sigma). Absorbance at 405 nm was measured at 30 minutes and 1 h.

8. *Discrimination between peptide and carbohydrate epitopes*

Identification of carbohydrate epitopes was carried out by testing reactivity in ELISA and immunoblot using infective *Neoparamoeba* antigen treated with 40 mM NaIO4 (Sigma, USA) in 50 mM acetate buffer pH 4.5 for 1 h at room temperature (RT). Antibodies demonstrating reactivity with both untreated and periodate treated antigens were considered to recognise peptide epitopes whereas loss of reactivity with periodate treated antigen was interpreted as indicating the recognition of carbohydrate epitopes (Woodward, 1985).

9. *SDS-PAGE analysis*

Antigens (4 to 9 μg/lane) with or without 100 mM dithiothreitol (DTT) were separated on SDS-PAGE gradients gels. Proteins were visualised by silver staining and Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain (Molecular Probes) was used for glycoprotein staining.

Native gel electrophoresis was performed using a NuPAGE Novex Tris-acetate gel (Invitrogen) and NuPAGE Tris-acetate running buffer (Invitrogen). High molecular weight markers (HMW calibration kit for native electrophoresis) were from Amersham.

10. ***Immunoblot Analysis***

Antigens (equivalent to 3 x 10^4 parasite/lane or 10 μg of protein) containing 100 mM dithiothreitol (DTT) were separated on SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, USA) for immunoblot analysis. Membranes were blocked in PBS containing 5% skimmed milk, washed and incubated for 2 h at room temperature with mouse serum diluted 1/100 or with tissue culture supernatant of selected hybridomas. Purified mouse IgG1 (MOPC-21, Sigma, USA) and purified mouse IgMκ (MOPC-104E, Sigma USA) were used as isotype controls for IgG and IgM respectively. The membranes were then incubated with alkaline phosphatase-conjugated second antibody (anti-mouse IgG, γ-chain specific and/or anti– mouse IgM, μ - chain specific; Sigma, USA) for 2 h at room temperature, washed and colour developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Sigma, USA).

11. *Indirect immunofluorescent-antibody test (IFAT)*

Parasites (1000 per sample) were heat fixed on glass slides, washed with PBS and incubated with 100 μL of hybridoma supernatant for 2 h at 37 ^oC. After washing, second antibody (FITC conjugated anti-mouse IgG, γ-chain specific or anti-mouse IgM, μ chain specific; Sigma, USA) diluted 1/100 in PBS-1% BSA was added and the slides incubated for 1 h at 37 ^oC. The slides were washed, mounted in FluorSave Reagent (Calbiochem) and visualised by fluorescence microscopy using a BP 520-550 excitation filter (narrow band width). Purified mouse IgG1 (MOPC-21, Sigma, USA) and purified mouse IgMκ (MOPC-104E, Sigma USA) were used as isotype controls.

12. *Flow cytometry*

Neoparamoeba spp parasites were incubated with hybridoma supernatants for 30 min in ice bath. Culture supernatant from NS1 cells and from hybridoma producing MAb specific for an irrelevant antigen were used as a negative controls. The parasites were washed twice with PBS-0.1% BSA, resuspended in anti-mouse IgM μ chain specific biotin conjugate (Sigma, USA) and incubated on ice for 30 min. Washed as above and incubated for another 30 min on ice with Streptavidin Alexa Fluor (Molecular Probes). After washing and resuspension in PBS-0.1% BSA, the parasites were analysed in a Becton Dickinson FACSCalibur flow cytometer using Cell Quest software.

13. *Confocal microscopy*

Parasites were fixed with paraformaldehyde 2% in PBS for 15 minutes, washed three times and incubated for 10 min in a slide pre-treated with 0.1% polyethylenimine (PEI). Cells were then permeabilised by incubation with 100 μL of cold methanol for 10 min, washed three times and blocked with PBS-10% FCS. One hundred microliters of hybridoma supernatant or purified MAb (10 μg/mL) were incubated for 1 h at room temperature in moist chamber. After washing, anti- mouse immunoglobulin Alexa fluor 488 conjugate (Molecular probes) diluted 1/300 in PBS was added and the slides incubated for 1 h at room temperature. The slides were washed, mounted in FluorSave Reagent (Calbiochem) and visualised using a confocal microscope Olympus Fluoview 300. Purified mouse IgMκ (MOPC-104E, Sigma USA) was used as negative control.

14*. Transmission electron microscopy (TEM)*

Wild type *Neoparamoeba* spp, (3×10^5 /ml) were collected in filtered sea water. Cells were centrifuged to obtain a visible pellet and fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1M PIPES buffer, pH 7.2 for 1h. Cells were washed in 0.1M Pipes, suspended in 1% low melt agarose at 55 ℃ and centrifuged immediately at 7000 rpm to obtain a pellet of cells in agarose. The agarose pellet of cells was cut into 2 small segments which were suspended in 0.1M PIPES buffer. The agarose pellets were dehydrated in a graded series of ethanol, infiltrated and embedded in LR White resin.
Ultra thin sections (70nm) were cut on an ultramicrotome (Reichert Ultracut E) and collected on 300 mesh Pioloform coated grids. Sections were incubated in 0.05M glycine in PBS for 15 min and then in blocking buffer (PBS-5% BSA-5% FCS) for 30 min. Sections were washed PBS-1% BSA and incubated in the primary antibody, MAb 44C12 in PBS-1%BSA, for 1h. After washing in PBS-1%BSA, sections were incubated in the secondary antibody, goat-anti-mouse IgM conjugated with 5nm gold particles, for 1h. Sections were washed in PBS-1%BSA and in PBS and post fixed in 2% glutaraldehyde in PBS for 5 min. After washing in PBS and distilled water, sections were stained with Uranyl Acetate (saturated aqueous) for 20min and lead citrate (Reynold's) for 4min. Sections were viewed using a Philips CM10 TEM and 100Kv. Purified mouse IgMκ (MOPC-104E, Sigma USA) were used as isotype controls.

15. *Immunohistochemistry*

Tissues were fixed in seawater Davidson's fixative (24 h), dehydrated through a graded ethanol series, cleared in xylene and infiltrated with paraffin. Sections were cut at 5 μm and attached to Polysine slides (Mezel-Glaser Braunschweig, Germany), dried overnight at 37 ˚C then de-waxed and re-hydrated. After a brief rinse in PBS (pH 7.4), sections were blocked for endogenous peroxidase (3% H_2O_2, 20 min), washed (PBS, 3 x 1 min) and incubated with normal horse serum (20 min, 20 ˚C) (Vector Laboratories). Sections were then blotted dry and incubated in a humid chamber (37 ^{o}C, 1 h) with 44C12 (1:1000). Sections were washed, then incubated (30 min, 20 ˚C, 1:500) with biotinylated horse anti-mouse IgG (Vector Laboratories), washed then incubated with peroxidase conjugated streptavidin (Invitrogen), washed and flooded with 3,3-diaminobenzidine (DAB) in peroxide buffer (2 min) (Roche Diagnostics), rinsed in dH_2O (30 sec), counterstained with Mayer's haematoxylin (20 sec), rinsed, differentiated in PBS (30 sec), dehydrated, cleared and mounted. Purified mouse IgMκ (MOPC-104E, Sigma USA) was used as isotype controls.

16*. Purification of HMWA by size exclusion HPLC.*

A Superdex 200 PC 3.2/30 column (Amersham Pharmacia Biotech) was used for the fractionation of the soluble fractions of infectious *Neoparamoeba* spp and non-infectious *N. pemaquidensis* PAO27 on an SMART chromatography system (Amersham Pharmacia Biotech). Sixty to 70 μg of soluble fraction were injected per run in the column equilibrated with 20mM ammonium acetate solution pH 7. Eluting protein peaks were analysed at 215nm and 280nm. Fractions of 120μL were collected with a flow of 40μL/min.

17. *Fractionation of HMWA by HPLC*

Ion exchange chromatography – WT HMWA was separated using a MonoQ column in 20mM Tris-HCL, pH 8.0 with a gradient of 0 to 100% 1M NaCl over 40 min. Fractions collected from MonoQ separation were used to coat an ELISA NUNC maxisorp plate and probed with the following antibodies: MAb CS-56(specific to chondroitin sulphate), MAb 97H9 (specific to fish mucus), MAb 44C12 and MAb 63C4. The same fractions obtained from MonoQ were dialysed and then used in DMMB assay at pH 1.5 and pH 3 (see 20)

Reverse phase chromatography (RP-HPLC) - TFMS deglycosylated WT HMWA (see 18) was further analyzed by RP-HPLC with a C4 column. Mobile phase used was a gradient of 2% acetonitrile/ 0.2% formic acid to 80% acetonitrile/ 0.2% formic acid over 60 min at flow rate of 100 μL/ min. Absorbance was monitored at 220 and 280 nm. Four major peaks were eluted from the RP-HPLC, which were subjected to tryptic digest and MS analysis.

18*. Chemical deglycosylation* (TFMS)

The HMWA (150 μg) was deglycosylated using the GlycoProfile IV, Chemical Deglycosylation Kit (Sigma) using Anisole as scavenger and 2 h of trifluoromethansulfonic acid (TFMS) treatment at 0 ^{o}C. The samples were dialysed against 20 mM ammonium acetate buffer pH 7 and concentrated to less than 10 μl using the speedy vac and run, under reducing conditions on 4-12% Tris gradient gel using MES buffer (Invitrogen)

19*. Glycosidase treatment of HMWA.*

PGNase F, neuraminidase and O-glycosidase - Four aliquots (9 μg/tube) of HMWA from infectious *Neoparamoeba spp* or IgM TEPC control (Sigma) were resuspended in 20 mM Phosphate buffer pH 7.2. One aliquot was treated with 3 U of PGNase F (Roche) alone, another one with 3.75 mU of O-glycosidase (Roche) + 3 mU of neuraminidase (Roche). The 3rd one was treated with all 3 enzymes and one aliquot remain untreated. The total reaction volume was 100 μl. After overnight incubation at room temperature samples were stored at -80^{o}C.

PNGase A and PGNase F - Aliquots (4ug/tube) of HMWA from infectious *Neoparamoeba* spp or Human IgA control (Bethyl Lab) were used in glycosidase treatment experiments. Samples for PNGase A (Roche) were resuspended in 20 mM Sodium acetate pH 5.1/ 1M NaSCN/ 0.2 M β-mercaptoethanol (β-ME) with the addition of 0.75mU PNGaseA. Samples for PNGaseF (Roche) were resuspended in 40 mM Sodium phosphate pH 7.2/ 0.2 M β-ME with the addition of 2.5U of PNGase F. Untreated samples were resuspended in the PNGase F buffer without addition of any enzymes. All samples were incubated for 24 hours at 37^{o}C.

All samples were dialyzed against 20mM ammonium acetate pH 7.0 prior to SDS-PAGE and immunoblot analysis.

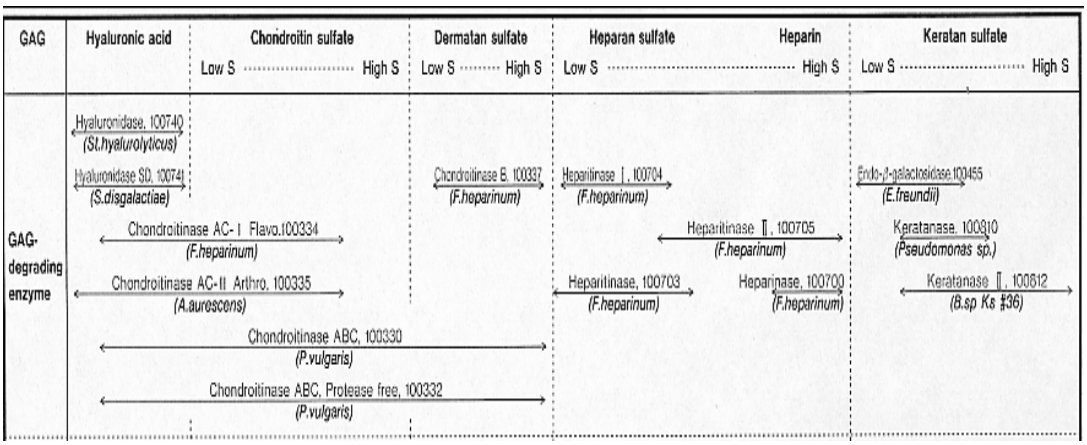
Enzymatic Protein Deglycosylation Kit (Sigma, # E-DEGLY) - WT HMWA was boiled in the presence of 10% SDS for 10 min followed by reduction and alkylation with tributylphosphine (TBP) and acrylamide. The reduced and alkylated HMWA was then desalted using BioRad Biospin columns followed by enzymatic deglycosylation. Briefly, the HMWA and fetuin (control) were resuspended in reaction buffer and denaturation solution and heated at 100 ^{o}C for 5 min. Samples were then cooled to room temperature and TritonX-100 added. 0.5 µL of each of the enzymes were added as follow and reactions incubated for 16 h at 37 ^{o}C. Reaction tube 1 – no enzyme added, Reaction tube 2 - PNGase F, Reaction tube 3 – O-glycosidase + α - 2(3,6,8,9)-neuraminidase + β-1,4-galactosidase + β-N-acetylglucosaminidase, Reaction tube 4 – all enzymes as above
Samples were analyzed by SDS-PAGE followed by glycoprotein stain or immunoblot with 44C12.

20. Identification and quantitation of glycosamino glycans (GAG)

DMMB assay – Sulphated GAG were identified and quantified using 1, 9-dimethylmethylene blue (DMMB) dye test (Farndale 1986) using an anion exchange purified bovine nasal cartilage proteoglycan (BNC-PG) as a standard. The assay was performed at pH 1.5 and 3 on one occasion to differentiate between carboxyl and sulphate groups. At pH 1.5, carboxyl groups are protonated and hence will bind less to DMMB therefore the assay performed at this pH predominantly measures sulphate groups.

Chondroitinase ABC and keratanase - WT HMWA and BNC-PG (positive control) were incubated with chondroitinase ABC (Seikagaku) and keratanase (Seikagaku) in 0.1M Tris pH6.5 at 37^{o}C water bath for 21 hours followed by DMMB assay at pH1.5
The samples were then analysed by SDS-PAGE followed by silver staining or immunoblot probed with 44C12 and 97H9 MAb.

Heparitinase – WT and PAO27 HMWA, PNC-PG (positive control), and heparin sulphate (positive control) were treated with heparitinase and heparitinase II (both from Seikagaku) in 50mM sodium acetate pH7.0, 5mM $CaCl_2$ at 37^{o}C water bath for 21 h followed by DMMB assay at pH1.5. The samples were then analysed by SDS-PAGE followed by silver staining or immunoblot probed with MAb 44C12 and MAb 97H9.



21. *Glycan array analysis*

MAb 44C12 was used to probe the printed glycan arrays version 2 (Blixt, 2004) following the standard procedure of Core H of the Consortium for Functional Glycomics (www.functionalglycomics.org/static/consortium/).

22. *Phospholipase C treatment of WT parasites*

Four aliquots of 8 x 10^4 WT parasites were washed twice with two times concentrated PBS and resuspended in 100 μL of Phospholipase C (PLC, Sigma) diluted in the same buffer. Two of the four tubes were resuspended in 0.08 units of PLC and the other two in 0.05 units of PLC. One tube at each concentration of FLC was incubated for 1 h at room temperature and the other incubated for 1 h at 37^{o}C. After treatment parasites were washed with PBS and reactivity with MAb 44C12 assessed by IFAT.

23. *Salmon immunization experiments*

1. Immunization with HMWA

An immunisation/challenge experiment was undertaken at University of Tasmania to analyse the immunogenicity of the HMWA in salmon and assess the potential of this preparation to protect immunised salmon from challenge with infectious *Neoparamoeba* spp. The trial followed the following format, with all immunisations being via the i.p. route:

Group 1: Immunisation test. 13 salmon (150 g) primed with WT-derived HMWA (50μg protein) emulsified in Freund's Complete Adjuvant (FCA) and boosted at day 35 with 50 μg of HMWA in Freund's Incomplete Adjuvant (FIA).
Group 2: Immunisation control. 17 salmon immunised with PBS emulsified in FCA (priming) or FIA (booster)
Group 3: Infection control. 17 salmon injected with PBS alone.

Serum and mucus samples were taken at days 0, 35 and 49 and the fish were challenged with *Neoparamoeba* spp at day 66. The experiment was terminated on day 105 when surviving fish were sacrificed and sampled for blood and mucus. The AGD status of the surviving fish was determined by histological examination of gill tissue.

2. Immunization with Freund's Adjuvant

An immunisation/challenge experiment was performed at University of Tasmania to assess the potential of FCA and FIA alone to protect salmon from challenge with infectious *Neoparamoeba* spp. The trial followed the following format, with all immunisations being via the i.p. route:

Group 1: 40 salmon (100 g) primed with PBS emulsified in FCA and boosted at day 35 with PBS in FIA.
Group 2: 40 salmon primed and boosted with PBS emulsified in FIA.
Group 3: Infection control. 40 salmon injected with PBS alone.

Fish were challenged with *Neoparamoeba* spp at day 66. The experiment was terminated on day 102 when surviving fish were sacrificed. The AGD status of the surviving fish was determined by histological examination of gill lesions.

3. Comparison of route of immunization in the induction of a mucosal immune response.

Two immunization experiments were performed at UTS to compare the effectiveness of i.p./ip. and i.p./spray immunization regimens with respect to their ability to elicit a mucosal antibody response.

Experiment 1:

Group 1 – Salmon (300 to 500 g) were primed by i.p. inoculation with 100 μg PA027 sonicated antigen emulsified in FCA and boosted at day 49 with 100 μg of the same antigen emulsified in FIA.
Group 2 – Primed by i.p. inoculation with 100 μg of PAO27 sonicated antigen emulsified in FCA and boosted at day 49 by spray immunisation with 100 μg soluble PAO27 sonicated antigen per gill.
Group 3 – Primed and boosted by spray immunisation with 100 μg of sonicated PAO27 antigen per gill.
Group 4 – Control. Primed by i.p. inoculation with PBS emulsified in FCA and boosted by spray immunisation with PBS alone at the gills.

Blood, gill mucus and skin mucus samples were collected on day 0, 29, 49, 80 and 117 post immunisation. Mucus samples were collected by gently scraping the skin or the gills with a cotton swab which was then placed in PBS with sodium azide and complete protease inhibitor cocktail (Roche). Protein concentrations were determined for all mucus samples and antibody concentration values normalised to protein content.

Experiment 2:

Group 1 – Salmon (100 to 150 g) were primed by i.p. inoculation with 100μg PA027 sonicated antigen emulsified in FCA and boosted at day 28 with 100μg of the same antigen emulsified in FIA.
Group 2 – Primed by i.p. inoculation with 100 μg of PAO27 sonicated antigen emulsified in FCA and boosted at day 28 by spray immunisation with 100 μg of soluble PAO27 sonicated antigen per gill.
Group 3 – Control. Primed by i.p inoculation with PBS emulsified in FCA and boosted by spray immunisation with PBS alone at the gills.

Blood and skin mucus samples were collected on days 0, 28, 56, 70 and 84 post immunisation. Mucus samples were collected by gently scraping the skin or the gills with a cotton swab which was then placed in PBS with sodium azide and complete protease inhibitor cocktail (Roche). Protein concentrations were determined for all mucus samples and antibody concentration values normalised to protein content.

RESULTS AND DISCUSSION

1. Production and selection of hybridomas (Villavedra, 2007).

To identify and characterise surface proteins involved in the attachment of the parasite to the gill epithelium we produced MAbs with enhanced reactivity to infective parasites using a subtractive immunisation method.

Mice were immunised with non-infective parasites and then treated with cyclophosphamide, a cytotoxic drug, to deplete reactive lymphocytes. This cycle was repeated once or three times and then mice were immunised with either whole infective parasites, or with untreated or deglycosylated membrane preparations from infective parasites. Spleen cells from immunised mice were harvested for cell fusion and hybridoma production and selection.

Results

No antibody responses to PAO27 antigen were detected by ELISA or immunoblot in the immunodepleted mice during the period when the cycles of immunodepletion were performed (Fig 2 and Fig 4 Panel A Lanes 2 and 5). The control mice (immunised with PAO27 but not immunodepleted) showed specific antibody responses by ELISA from day 14 (3 fold increase in O.D. with respect to day 0) reaching values of 2.7 OD units (14 fold increase in O.D. with respect to day 0) over the next 2 weeks (Fig. 2). The immunoblot profile of the immunised control mice showed multiple and defined bands across a wide range of *M*r (Fig 4, Panel A, lane 12).

After boosting with WT antigen, the immunodepleted mice showed increased levels of WT antigen-specific antibody as indicated by a 6 fold increase in O.D. values in the ELISA (Fig 3). Immunoblot analysis of the sera from these mice after boosting with WT parasite antigen showed that although they still reacted with PAO27 parasite antigens (Fig 4, Panel A, lanes 3 and 6) they recognised fewer antigen bands with an overall poorer response compared to the sera from mice immunised with the same antigens but not immunodepleted (Fig 4, Panel A, lanes 8 and 10). On the other hand the response of the immunodepleted mice was stronger to high molecular weight WT antigens (Fig 4, Panel B). In all cases the response to whole WT antigens showed a smear typical of heavily glycosylated molecules. Only a few discrete bands could be seen in the mice immunodepleted but not immunised. No differences were observed in the immunoblot profiles of the mice immunised with whole WT or membrane preparation.

When whole untreated WT parasites were used to boost the immune response in non-immunodepleted mice 27% of MAbs arising from fusion of isolated spleen cells were specific for WT parasite antigens, but only 5% (1 in 21) were specific for surface antigens as indicated by reactivity in IFAT. When subtractive immunisation was used the proportion of WT specific MAbs increased to 86% as did the percentage of MAbs specific for surface antigens (14%) but all of these MAbs recognised carbohydrate epitopes (Table 2).

When deglycosylated WT membrane preparations were used to boost the immune response, the number of MAbs specific to surface antigens increased from 5% to 38% compared to an increase from 14% to 20% when subtractive immunisation was

used, but again only 2 MAbs from this last fusion could be considered specific to non-carbohydrate epitopes (Table 2).

When subtractive immunisation was used in combination with boosting with untreated WT membrane, the overall number of MAbs increased dramatically as well the number of MAbs specific to surface molecules (50 out of 150) but again all these MAbs were specific to carbohydrate epitopes (Table 2). The total number of MAbs recognising carbohydrate epitopes on the surface of the wild type parasites from all fusions performed was very high (97 %) (Table 2). In addition, the large majority of the MAbs produced were of the IgM isotype. The use of MPL–TDM adjuvant for the positive immunisation increased the percentage of IgG MAb produced, but again all the IgG MAb produced were specific to carbohydrate epitopes. The characteristics of all the MAbs specific to surface molecules produced during this project are listed in Table 3.

When the MAbs were analysed by immunoblot, those considered specific to non-carbohydrate epitopes by ELISA did not react with the WT antigens (data not shown) suggesting that these MAbs recognise conformational peptide epitopes. Similarly, 41% of the MAbs specific for carbohydrate epitopes by ELISA and IFAT also did not react with immunobloted WT antigens (data not shown). The remaining carbohydrate specific MAbs reacted with immunoblotted WT antigens producing patterns of multiple bands or smears which is indicative of the presence of highly glycosylated antigens (Fig 5). Only a few MAbs showing a smeared pattern on immunoblot were reactive to material obtained from the gills of non-infected fish (data not shown).

The glycoprotein staining of the membrane preparations showed a smear across the whole range of *M*r (Fig. 6 lane 3). Deglycosylation of the membrane preparations using PNGase F and O- glycosidase alone did not result in an observable effect on the glycoprotein staining pattern (Fig. 5 lane 1 and 3), while the sample treated with PNGase F, O-glycosidases and neuraminidase showed significant deglycosylation with the exception of four bands above 160 kDa that were resistant to this enzymatic deglycosylation (Fig. 6 lanes 2 and 3).

Discussion

The lack of a detectable antibody response to non-infective parasites in the mice subjected to subtractive immunisation demonstrated that the immunodepletion protocol was effective, but the response to PAO27 antigens after boosting with WT antigens highlighted the short duration of this immunodepletion.

Membrane preparations were used in an attempt to increase the number of MAbs to surface molecules and at the same time the antigen was deglycosylated to increase the likelihood of obtaining MAbs to peptide epitopes. The result was that the overall numbers of MAbs to WT specific antigens decreased. The deglycosylation procedure although effective in decreasing the level of glycosylation observed in SDS-PAGE had little or no effect on the yield of MAbs specific for peptide epitopes. Even though the deglycosylation of the membrane was not complete and hence some response to carbohydrate epitopes was expected, the lack of response to peptide epitopes was unexpected. Furthermore, the high number of MAbs specific for carbohydrate epitopes expressed on the surface of the parasite obtained when deglycosylated membrane was used as immunogen, suggests that the majority of the surface molecules unique to WT parasites are carbohydrate in nature.

The possibility of destruction of a significant number of peptide epitopes by the periodate oxidation technique is unlikely since mild periodate oxidation performed at acidic pH has been shown to affect only carbohydrate moieties (Woodward, 1985). In fact, the large majority of the MAbs exhibiting cross-reactivity between the cultured and WT parasites recognised epitopes that were not susceptible to periodate oxidation (data not shown), as did the majority (80%) of the cross-reactive MAbs obtained after immunisation with non-infective parasites (Villavedra, 2005). These results taken together suggest that the dominant epitopes expressed on the surface of the infectious WT parasites are carbohydrate in nature and the common epitopes shared between infectious and non-infectious (PAO27) parasites are most probably peptide in nature.

The smears and multiple bands obtained by immunoblot with both the sera from WT antigen immunised mice and some of the MAbs, is characteristic of the profile expected for heavily glycosylated molecules (Vongchan, 2005). The fact that the MAbs recognise molecules present in the glycocalyx of the parasite does not necessarily mean that all the molecules are of parasite origin. Some of the MAbs showing a smeared pattern on immunoblots (e.g. 97H9; Fig. 5, lane 7) recognise the same epitope in WT antigen and material scraped from the gills of non-infected fish (data not shown) which means that the MAb is specific for host derived molecules associated with the parasite glycocalyx. Alternatively, this MAb recognises a cross-reactive epitope present on antigens expressed by the parasite and the host.

Table 1: Summary of the protocols followed in the fusions performed

Fusion No	Immune Depletion	Antigen and day of priming	Antigen and day of 1st booster	Antigen and day of last booster
1	No	Whole WT parasites Day 0	----------	Whole WT parasites Day 30
2 & 3	Yes	Whole WT parasites Day 50	Whole WT parasites Day 64	Whole WT parasites Day 79
4 & 5	No	Whole WT parasites Day 0	Deglycosylated WTmembrane Day 30	Deglycosylated WT membrane Day 56
6 & 7	Yes	Whole WT parasites Day 51	Deglycosylated WT membrane Day 77	Deglycosylated WT membrane Day 93
8	Yes	Untreated WT membrane Day 23	Untreated WT membrane Day 42	Untreated WT membrane Day 66

Table 2: Summary of the results obtained from all the fusions performed.

	Immune Depletion	Antigen used for Last booster	WT-specific MAbs [no. WT positive / no. *Neoparamoeba* positive]	Cell surface-reactive WT-specific MAbs [no. surface specific WT positive/ no.WT positive tested]	
Fusion No			**Total**	**Total**	**Carbohydrate**
1	No	Whole parasites	21/79 (27%)	1/21 (5%)	1/1 (100%)
2 & 3	Yes	Whole parasites	57/66 (86%)	8/57 (14%)	8/8 (100%)
4 & 5	No	Deglycosylated Membrane	16/79 (20 %)	6/16 (38%)	6/6 (100%)
6 & 7	Yes	Deglycosylated Membrane	42/105 (40%)	9/46 (20%)	7/9 (78%)
8	Yes	Untreated Membrane	158/365 (43%)	50/150 (33%)	50/50 (100%)
		TOTAL		74/290 (26%)	**72/74 (97%)**

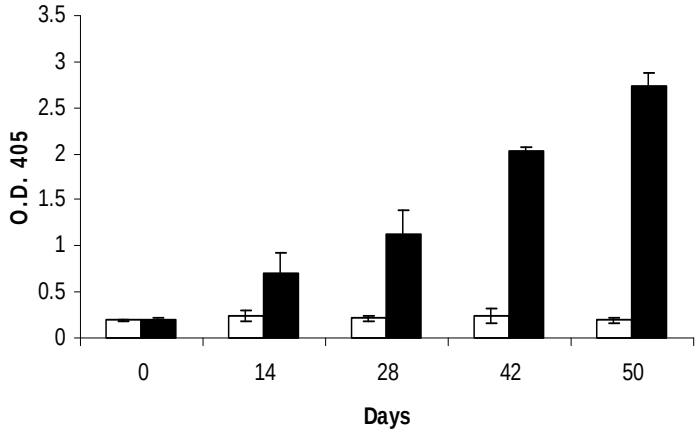


Figure 2: Antibody response to PAO27 parasites (determined by ELISA) of 8 PAO27/cyclophosphamide immunodepleted mice (white) and 3 non-immunodepleted mice (black). Results from two independent experiments.

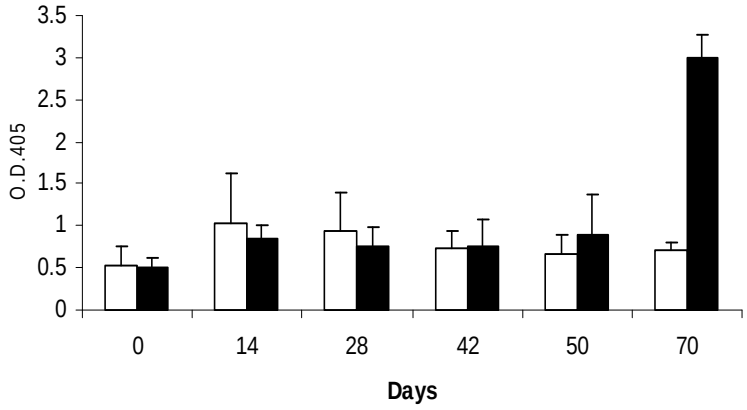


Figure 3: Antibody response to wild type parasites (determined by ELISA) of 8 PAO27/cyclophosphamide immunodepleted mice. Four mice from two different experiments were primed, at days 52 and 56 respectively, with wild type antigen emulsified with Freund's Incomplete Adjuvant (black). The remaining 4 were not immunised with wild type antigen (white).

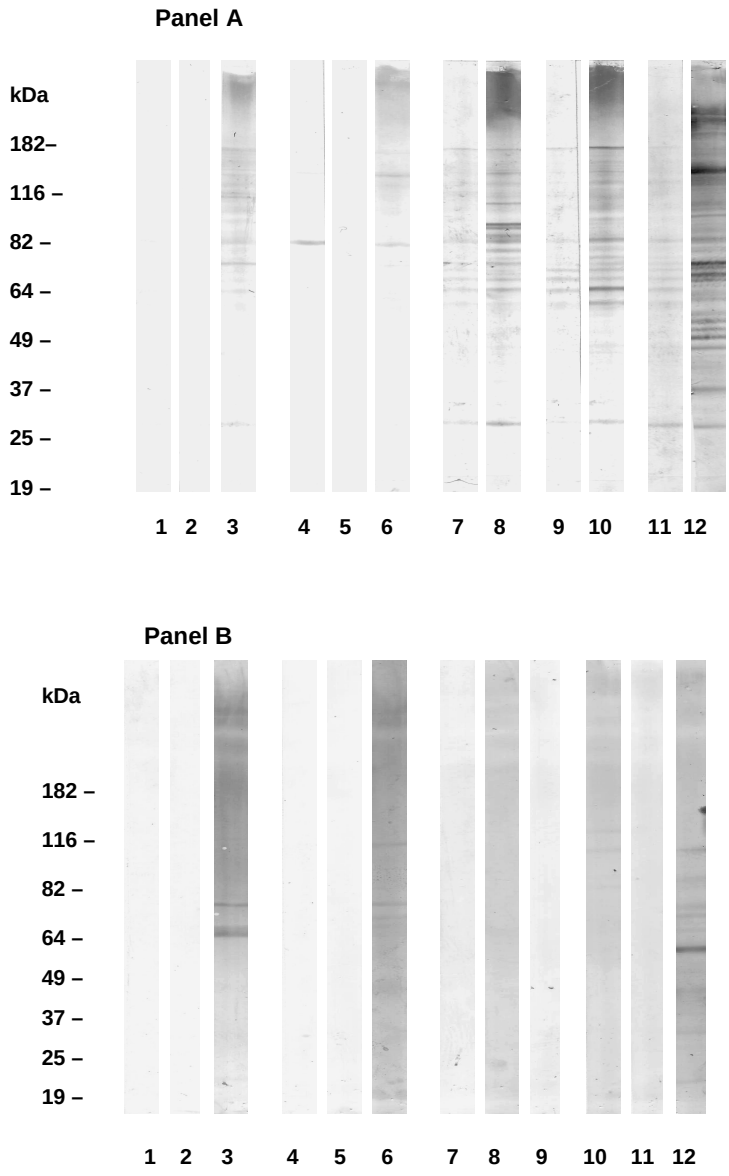


Figure 4: Immunoblot analysis of the polyclonal antibody response of mice to PAO27 parasite antigens (Panel A) and to WT parasite antigens (Panel B). Lanes 1, 4, 7, 9 and 11 – Day 0 of each mouse. Lanes 1 to 3 – PAO27/cyclophosphamide immunodepleted mouse immunised with membrane preparation from WT parasites. Lane 2 – day 23 (after 2 cycles of immunodepletion), Lane 3 – day 50 (after Priming and booster with untreated membrane). Lanes 4 to 6 – PAO27/cyclophosphamide immunodepleted mouse immunised with whole WT antigens. Lane 5 – day 50 (after 4 cycles of immunodepletion) and Lane 6 - day 100 (after priming with whole parasites and booster with deglycosylated membrane). Lane 7 and 8 – Non-immunodepleted mouse immunised with membrane preparation from WT parasites. Lane 9 and 10 – Non-immunodepleted mouse immunised with whole WT antigens. Lanes 8 and 10 – day 15 after priming. Lane 11 and 12 – non-immunodepleted mouse immunised with PAO27 antigens. Lane 10 – Day 50 (after 4 inoculations with PAO27 antigen but not immunodepleted).

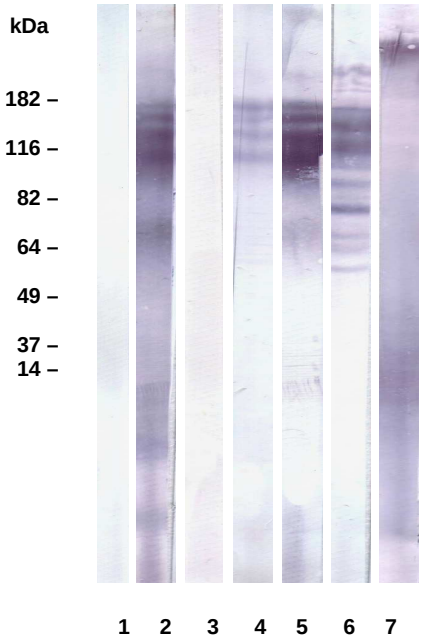


Figure 5: Immunoblot profiles of some of the WT surface antigen-specific MAbs recognising carbohydrate epitopes. Lane 1 – IgG isotype control – IgG kappa (MOPC21), lane 2 – MAb 115F1G7 (IgG), lane 3 - IgM isotype control – IgM kappa (TEPC183), lane 4 – MAb 98D10 (IgM), lane 5 – MAb 44C12 (IgM), lane 6 – MAb 116G11 (IgM), lane 7 - MAb 97H9 (IgM).

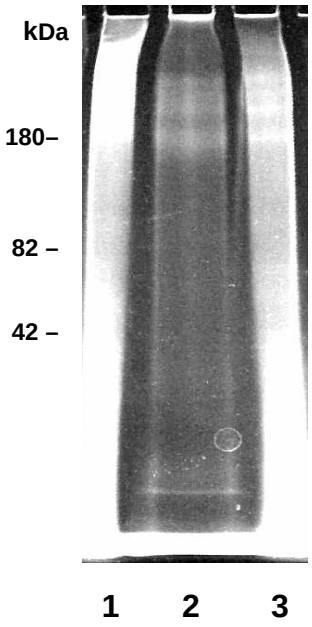


Figure 6: Glycoprotein stain after deglycosylation of a membrane fraction from freshly isolated parasites with: PGNase F and O – glycosidase (Lane 1); PGNase F, neuraminidase, and O – glycosidase (Lane 2); untreated membrane fraction (Lane3). Samples were separated in a NuPAGE Novex Bis-Tris 4-12% gradient gel using MOPS SDS as running buffer (Invitrogen).

Table 3 – Characteristics of surface antigen-specific MAbs.

Fusion	Hybridoma	MAb isotype	Specificity of MAb	Nature of epitope[2]	IFAT	Immunoblot pattern[3]
4	4C12	IgM	S	C	+++	HMWA Group 1
4	8G6	IgM	S	C	+	HMWA Group 1
4	8H4	IgM	S	C	+++	HMWA Group 1
4	7A1	IgM	S	C	++	HMWA Group1
4	5A6	IgM	S	C	+/-	*nd*
4	5E6	IgM	S	C	++	Negative
4	5F11	IgM	S	C	+/-	*nd*
4	6D6	IgM	S	C	++	HMWA Group 1
4	7G5	IgM	S	C	++	HMWA Group 2
4	8H11	IgM	S	C	+++	*nd*
4	5E2	IgG	X	P	++	*nd*
6	2H1	IgM	S	P/C	few +	*nd*
6	3C1	IgM	S	P	½ +	Negative
6	3C4	IgM	X	C	++	6kDa Group 7
6	1D2	IgM/IgG	S	C	½ ++	*nd*
6	3G5	IgM	S	C	+	Negative
6	2D8	IgM	S	C	+	Negative
6	1F6	IgM	S	C	++	Negative
6	1A10	*nd*	S	*Nd*	+/-	Negative
6	2D7	IgM	S	C	+	Negative
6	2G2	IgM	S	C	Few +	Negative
6	2F2	IgM	X	P		*nd*
6	3F2	*IgM*	S	P	++	*nd*
9	7H9	IgM	S	C	+++	HMWA Group 6
9	8H12	IgM	S	C	+++	*nd*
9	2B5	IgG	S	C	+++	HMWA Group 2
9	6A12	IgM	S	C	+++	HMWA Group 2
9	5H3	IgG	S	C	+++	Negative
9	8D10	IgM	S	C	+/-	HMWA Group 5
9	5E2	IgM	X	C	++	HMWA Group 2
9	1C5	IgM	X	*Nd*	+++	Negative
9	2D4	IgG	X	C	+++	Negative
10	3D7	IgM	X	P	+++	50 kDa Group 8
11	5F1	IgG	S	C	+++	HMWA Group 2
11	5H11	IgM	S	C	+++	*nd*
11	6A3	IgM	S	C	+++	*nd*
11	6G11	IgM	S	*Nd*	++	HMWA Group 3
11	6H9	IgG	S	C	++	Negative
11	9A1	IgG	S	C	+	Negative
11	9H10	IgG	S	C	++	Negative
11	11D9	IgG	S	C	+	Negative
11	1D9	IgM	S	*Nd*	+++	*nd*
11	5F10	IgM	X	*Nd*	+	*nd*
11	9A6	IgM	S	*Nd*	++	HMWA Group 3
11	2H1	*nd*	S	*Nd*	+/+	Negative
11	3A8	IgM	S	C	++	Negative
11	3B2	IgM	S	*Nd*	++	*nd*
11	4D1	*nd*	S	*Nd*	++	*nd*
11	4D2	IgM	S	*Nd*	++/+	*nd*
11	4F4	Nd	S	*Nd*	Few +	Negative
11	5E6	IgM	S	C	++	Negative
11	6D9	*nd*	S	?	+/+	Negative
11	6E1	IgM	S	C	++	Negative
11	6H12	IgM	S	C	+	HMWA Group 3
11	7C12	*nd*	S	*Nd*	few +	*nd*
11	8H3	IgM	S	C	+	Negative
11	10F11	IgM	S	C	+	*nd*
11	11F7	IgM	S	C	+	HMWA Group 3

Table 3: Explanatory notes

1. MAbs reacting specifically with infective, WT parasites are designated S; MAbs exhibiting reactivity with both WT and cultured PA027 parasites are designated X
2. The chemical nature of the epitope recognised by the MAb is indicated as either C for carbohydrate or P for peptide.
3. The molecular nature of the antigen(s) recognised by the MAbs was determined by immunoblotting performed on antigens fractionated by SDS-PAGE. The MAbs were grouped based on the pattern of antigens observed in the immunoblots.

2. Production of polyclonal antibodies

In addition to the WT specific MAbs we proposed to use polyclonal antibodies to identify and characterise surface proteins unique to WT parasites. Two strategies were used to obtain these polyclonal antibodies specific to infectious *Neoparamoeba* spp. The first consisted of immunising rabbits with a "WT enriched" antigen preparation which was comprised of WT antigen depleted of the majority of the antigens that are common to both non-infective PA027 and infective parasites. The second strategy was based on depletion anti-WT rabbit sera of antibodies that were cross-reactive with PAO27 antigens.

Depletion of WT antigen by affinity chromatography using anti-PAO27 antibodies.

The optimum ratio anti-PAO27 antibody:WT sonicated antigen for a maximum depletion was 14 mg of crude antibody/mg of WT antigen (Fig. 7). The various antigen fractions arising from this procedure were analysed by ELISA, SDS-PAGE (Fig 8) and immunoblot using anti-PA027 and anti-WT sera (Fig 9). The results show that while absorption on anti-PA027-Sepharose does remove some antigens that cross-react with anti-PA027 antiserum (Fig 8: lanes 3, 4 and 5) this depletion was not significant as the protein profiles of the unfractionated and WT antigen "enriched" preparations were not qualitatively different (Fig 8: lanes 1 and 2). This was confirmed by the immunoblot analysis (Fig 9) which showed that the unfractionated and WT "enriched" preparations exhibited the same reactivity profile when probed with anti-PA027 serum (lanes 6 and 7).

The antigen fractions eluted from the column at pH 2.5 represented only 10 to 20 % of the original protein loaded and it was therefore assumed that the balance of the protein was still bound to high affinity anti-PA027 antibodies on the column. Thus, two additional elution steps were performed using NaSCN and 40%dimethyl sulfoxide (DMSO). This harsh treatment enabled the recovery of all the bound antigen. However, subsequent use of the column gave anomalous results that we were able to show was due to a combination of aggregation of the antigen preparation and damage to the affinity column by the harsh washing procedures required to remove all the bound material. These findings meant that new affinity columns would have to be prepared for depletion of each batch of antigen, a process that was not practical or sustainable.

Depletion of cross-reactive antibodies using PAO27 antigen adsorbed onto PVDF membrane.

With the realisation that antigen depletion by the procedures outlined above was problematic due to the inherent nature of the antigen preparation (aggregation of membranous materials) and the harsh conditions required to strip the column, we commenced parallel experiments, employing a different strategy. A rabbit was immunised with whole sonicated WT (infective) parasites and antibodies cross-reacting with the non-infective PA027 parasites were removed by incubating the diluted rabbit serum with PA027 antigen adsorbed to PVDF membranes. The original antiserum and the PA027-adsorbed serum were analysed by ELISA against WT and PA027 antigens (Figures 10 and 11), which demonstrated that the PA027-depleted serum retained reactivity against WT antigen, but failed to react significantly with PA027 antigen. Furthermore, periodate treatment to destroy carbohydrate antigens revealed that the majority of the PA027 cross-reactive antibodies in the original anti-WT rabbit antiserum are directed against carbohydrate antigens expressed by the PA027 parasites (Fig 11, a). The apparent contradiction with previous results in terms of the carbohydrate nature of the WT specific epitopes can

be explained by the fact that in the previous section we selected and characterised MAbs that were specific to surface antigens while in this experiment we used polyclonal antibodies from a rabbit immunised with whole WT antigen. In addition, the different immunological responses of mice versus rabbits in terms of their recognition of dominant antigens could explain the apparent contradictory results.

Thus, by depletion of the original antiserum with PA027 antigens immobilized on PVDF membrane we generated a polyclonal antibody reagent that is essentially specific for WT infective parasites. Unfortunately, because of problems that subsequently arose with the *in vitro* parasite attachment assay, this depleted antiserum was not tested for its ability to block the attachment of parasites to salmon gill epithelium.

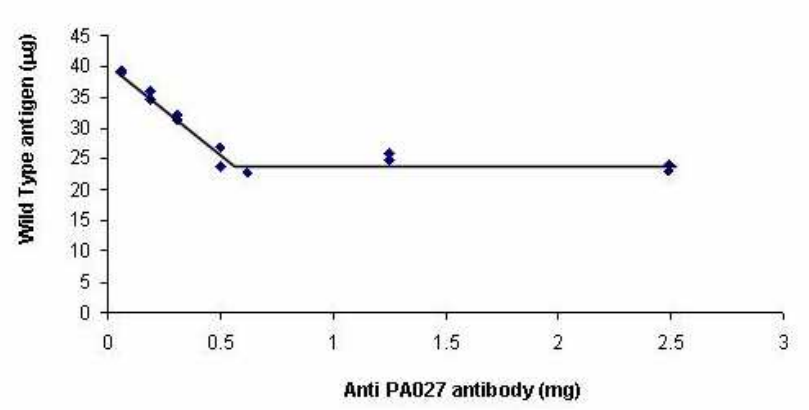


Figure 7: Protein concentration of the supernatant obtained after the incubation of 40 µg of WT antigen with different amounts of crude immunoglobulin fraction of anti-PA027 rabbit serum coupled to CNBr-Sepharose. The optimum ratio of antigen: immunoglobulin fraction for the depletion of the cross-reactive antigens between WT and PA027 parasites is around 0.55 mg for 40 µg (13.75mg Antibody/mg) of WT antigen.

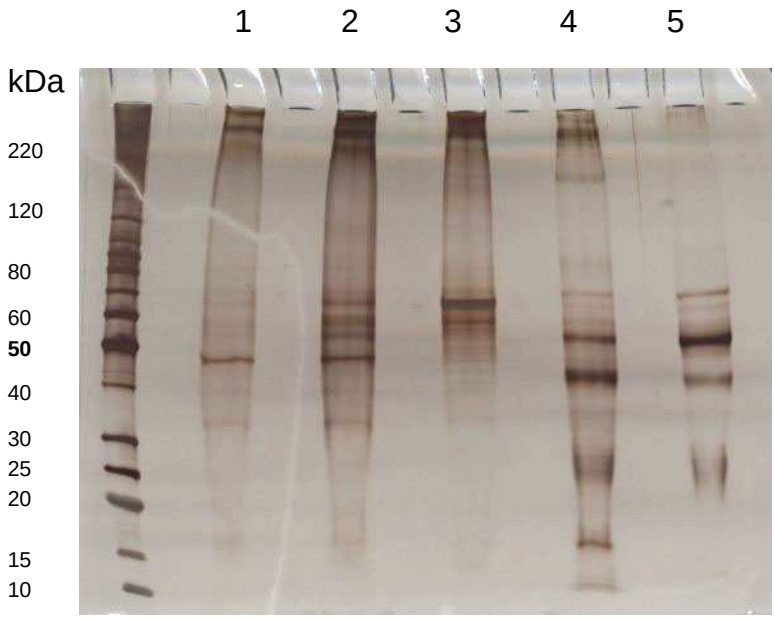


Figure 8: Analysis of WT antigen depletion by SDS-PAGE. Samples from WT antigen depletion experiment were analysed by SDS-PAGE under reducing conditions followed by silver staining. Lane 1 - total WT antigens (original unfractionated preparation), lane 2 – fraction enriched for WT specific antigens (depleted of cross-reacting antigens by absorption with anti-PA027-Sepharose), lane 3 – fraction eluted at pH2.5, lane 4 – fraction eluted with NaSCN, lane 5 –fraction eluted with Gly/DMSO.

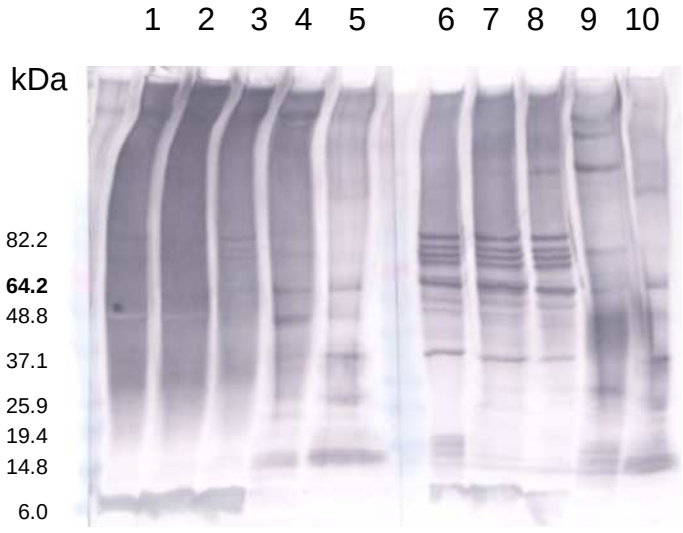


Figure 9: Immunoblot analysis of WT antigen depletion. Samples were analysed by SDS-PAGE in a 4-20% gradient gel under reducing conditions followed by immunoblot. lanes 1 & 6 - WT antigens (original unfractionated preparation), lane 2 & 7 - fraction enriched for WT specific antigens (depleted of cross-reacting antigens by absorption with anti-PA027-Sepharose), lanes 3 & 8 - fraction eluted at pH2.5, lanes 4 & 9 - fraction eluted with NaSCN, lanes 5 & 10 - fraction eluted with Gly/DMSO. Lanes 1-5 were probed with rabbit anti-WT serum, lanes 6-10 were probed with rabbit anti-PA027 serum.

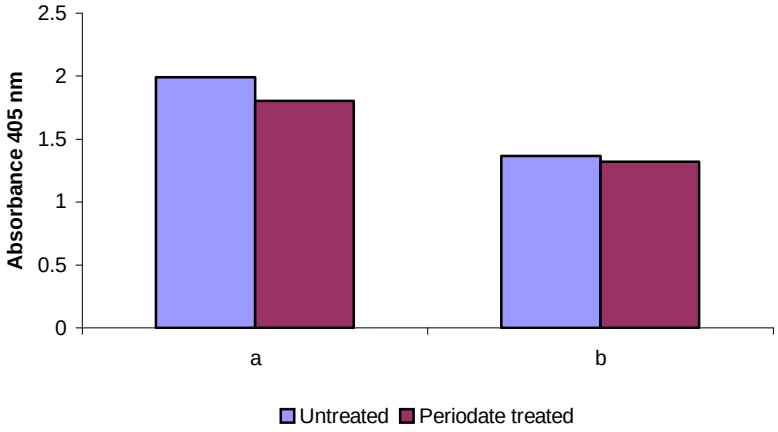


Figure 10: ELISA showing reactivity of rabbit anti-WT parasite antiserum with WT antigen. The rabbit antiserum (a), or antiserum absorbed by passage over immobilised PA027 antigen (b), was assessed for binding activity against both untreated and periodate-treated WT antigen. Binding of rabbit antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG. The data is the average of values obtained from analysis of duplicate samples.

Figure 11: ELISA showing reactivity of rabbit anti-WT parasite antiserum with PA027 antigen. The rabbit antiserum (a), or antiserum absorbed by passage over immobilised PA027 antigen (b), was assessed for binding activity against both untreated and periodate-treated PA027 antigen. Binding of rabbit antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG. The data is the average of values obtained from analysis of duplicate samples.

3. *In vitro* attachment assay.

Two polyclonal rabbit sera and some selected MAbs recognising antigens expressed on the surface of the parasite were tested for their capability to block the attachment of the parasite to salmon gill epithelium in an *in vitro* attachment assay developed at TAFI, University of Tasmania (Nowak, 2004). The MAbs that inhibit parasite attachment were then used to identify and characterise attachment-associated molecules.

Results

Six MAbs were tested in the *in vitro* parasite attachment assay developed at University of Tasmania. Three MAbs (44C12, 47A1 and 48G6) exhibited statistically significant levels of inhibition of the attachment of parasites to gill explants when compared to the negative isotype-matched control antibody (Fig. 12 and Table 4) showing levels of inhibitory activity ranging from 51 to 77% with respect to the isotype control (Table 4). Statistical analysis of the results was performed using Kruskal-Wallis Non-Parametric Test and Dunn's Multiple Comparison Test using GraphPad Prism program version 4.03 (Table 5).

MAbs 63C4 and 103D7 were tested twice with contradictory results. On the first occasion both MAbs showed clear inhibitory activity but the data were not sufficient to show significant differences in the statistical analysis. The second testing of these MAbs showed no blocking activity (data not shown).

Two rabbit polyclonal antisera shown to be reactive with *Neoparamoeba* spp by ELISA and IFAT were tested in the *in vitro* attachment assay. The first serum was from a rabbit immunised with sonicated WT parasites; the second was from a rabbit immunised with a membrane preparation from WT parasites. A negative control serum (day 0 or pre-bleed from the second rabbit) was also tested. The anti-membrane antiserum was tested twice and the anti-whole parasite antiserum three times, along with a seawater (no antiserum) control and the negative control serum. There were no significant differences in the ability of these antisera to block attachment of the parasite to gill explants when compared to the negative control (data not shown).

Discussion

MAbs 44C12, 47A1 and 48G6 (Group 1 MAbs) all exhibited a statistically significant capacity to inhibit the binding of infective parasites to gill epithelium when compared to no antibody (i.e. sea water only) and an isotype matched antibody control. The consistency of this result was highlighted by the fact that the three MAbs all recognise the same high molecular weight antigen pattern on immunoblots. A fourth antibody, 48H4, that recognises the same antigen pattern exhibited statistically significant inhibition of attachment compared to the no antibody control, however statistical significance compared to the isotype matched control was not demonstrated.

Subsequent testing of two MAbs, 63C4 and 103D7 that recognise antigens distinct from the HMW pattern typified by Group 1 MAbs revealed high levels of variation in the results obtained such that statistical analysis could not be performed. These results, together with testing carried out on two polyclonal antisera, revealed levels of inconsistency in the assay that indicated a change in the performance of the assay over a period of several months. Dr. Powell at University of Tasmania undertook a histological examination of tissue samples from later experiments in this series and

showed necrotic tissue in all the gill explants tested after 24 h incubation with or without antibody present. This observation would certainly explain the highly variable results obtained in these assays and may explain the differences in the performance of assays undertaken in the first half of 2005 and those carried out at the end of the same year. Whatever the explanation, the ongoing validity of the *in vitro* attachment assay as a means of assessing potential targets for vaccine development had to be questioned. Clearly, further development and validation of the assay was required if it was to be used as a go/no go assessment. Such development work was beyond the scope of the current project. However, the fact that the three Group 1 MAbs, recognising the same antigen pattern in immunoblot, were independently tested and showed statistically significant levels of inhibition of attachment in the assay compared to appropriate controls allowed some level of confidence in the initial assay results and, as a consequence, characterization of the antigens recognised by the these MAbs was continued.

Table 4 – Summary of attachment assay results.

Group		Fusion #	MAb	Isotype	Specificity	Nature epitope	IFAT	% Inhibition attachment assay
1	HMWA	4	**44C12**	IgM	S	C	+++	51%
		4	48G6	IgM	S	C	+	53%
		4	48H4	IgM	S	C	+++	53%
		4	47A1	IgM	S	C	++	77%
		4	46D6	IgM	S	C	++	*nd*
7	6 kDa	6	**63C4**	IgM	X	C	++	*nsi*
8	50 kDa	10	**103D7**	IgM	X	P	+++	*nsi*

X – cross-reactive MAb ; S – specific MAb; C – carbohydrate epitope; P – peptide epitope; nd – not determined;
nsi – no significant inhibition; HMWA – High molecular weight fraction.

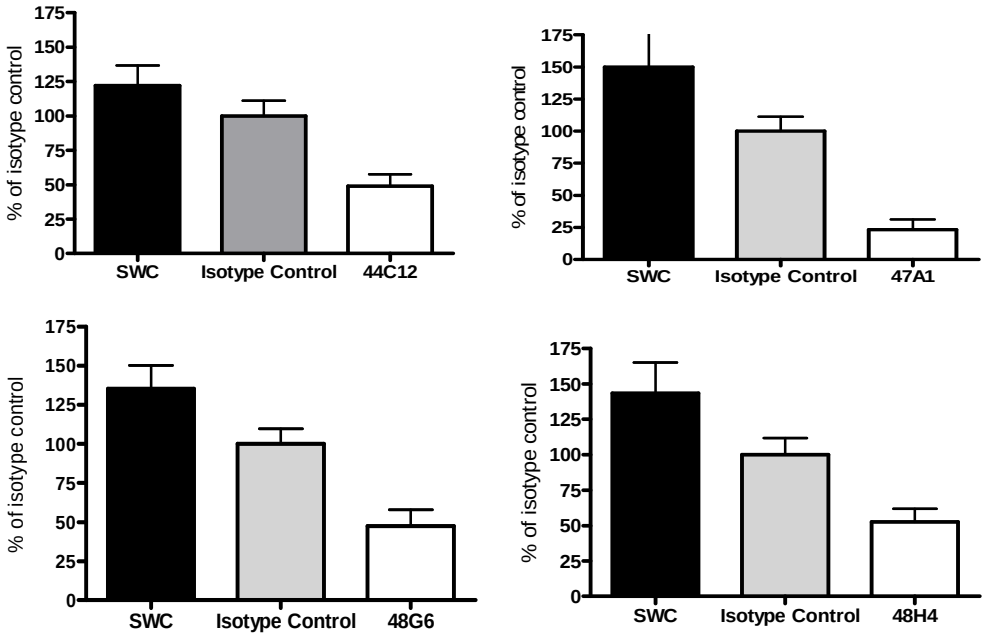


Figure 12: Percent parasites attached to gill explants, compared to the isotype control, after pre-incubation of WT parasites with 4 MAbs from Group 1 (44C12, 47A1, 48G6 and 48H4). The results are the mean & SEM from 2 or 3 *in vitro* attachment assays.

Table 5 - Statistical Analysis of the results from the *in vitro* attachment assay using Kruskal-Wallis Non-Parametric Test and Dunn's Multiple Comparison Test

44C12	SWC vs. Isotype control	P > 0.05	ns
	SWC vs. 44C12	P < 0.001	***
	Isotype control vs. 44C12	P < 0.01	**
48G6	SWC vs. Isotype control	P > 0.05	ns
	SWC vs. 48G6	P < 0.001	***
	Isotype control vs. 48G6	P < 0.01	**
47A1	SW control vs. Isotype control	P > 0.05	ns
	SW control vs. 47A1	P < 0.001	***
	Isotype control vs. 47A1	P < 0.01	**
48H4	SW control vs. Isotype control	P > 0.05	ns
	SW control vs. 47A1	P < 0.01	**
	Isotype control vs. 47A1	P > 0.05	ns

4. Inhibition of infection assay

In an attempt to confirm and extend the results obtained initially in the *in vitro* attachment assay we investigated an assay to test the inhibition of parasite attachment *in vivo.* This assay was based on an infection model already established at University of Tasmania in which defined numbers of parasites were introduced to susceptible fish in a controlled tank environment. In order to test the ability of selected antibodies to inhibit the binding of parasites to gill tissue *in vivo*, infective parasites were pre-incubated with the antibodies prior to their introduction to the tank. This assay had a number of challenges. In addition to the fact that antibodies usually don't remain bound to the surface of a live target cell for an extended period of time, *Neoparamoeba* multiply (sometimes doubling) in 24 h. Therefore, to adapt the infection model for use as an inhibition of infection assay, it was necessary to reduce the time of exposure of the parasites to the fish to ensure that the MAb remained bound to the surface of the parasite during the entire exposure period. At the same time it was important to obtain a certain minimum number of parasites attached to the gills in order to assure statistical significance. Dr Phil Crosbie and Dr Mark Adams had been working at University of Tasmania on the adaptation of the infection model to these conditions (Crosbie, 2007).

Since the parasites could shed or endocytose antibodies bound to their surface it was necessary to test the stability of the antibodies on the surface of parasites *in vivo* under infection conditions (1×10^4 suspended in 300 L of seawater). We therefore performed a set of experiments where aliquots of live WT parasites (1×10^5 in 1.5 ml of seawater) were incubated with the MAb 44C12, MAb 97H9 or the isotype control in PBS for 30 min and then diluted to 300 mL in seawater. After 6, 12 and 24 h of incubation in seawater the parasites were recovered by centrifugation at 10000 g for approximately 8sec. Two aliquots of the parasites were also incubated in PBS with the antibodies (MAb or isotype control) but were not incubated in seawater. These samples represented the 0h or maximum binding level controls. To assess the level of MAb binding following varying time periods of incubation the parasites were incubated with anti-mouse IgM-FITC conjugate and visualised under a fluorescence microscope.

To establish if any particular MAb remained associated with the surface of the parasite in seawater we also tested the reactivity of MAbs 115F1, 47G5, 44C12, 92B5, 47A1 and 98D10 to parasite antigen in seawater by ELISA.

Results

While the recovery of parasites was satisfactory for the majority of the time points tested, there were no positively stained parasites observed at any of the time points, including the 0 h control samples.

ELISA testing of the stability of MAbs in seawater showed only MAb 97H9 showed any reactivity with parasite antigen after 30 min incubation in seawater. (Fig13). The reactivity of all the other MAbs dropped dramatically at the 30 min time point.

Discussion

The failure to detect antibodies bound to the surface of parasites in the 0 h control samples was unexpected. MAbs 44C12 and 97H9 showed strong reactivity to the surface of the parasite by IFAT. However, we observed very low and inconsistent signals in flow cytometry assays with these antibodies using a staining protocol very similar to that used for the 0h control in this experiment. One possible explanation for this apparent paradox is that live parasites suspended in a hypo-osmotic solution (PBS), shed their glycocalyx and therefore become unreactive with MAbs specific for carbohydrate epitopes expressed on glycoproteins in the glycocalyx. These results are consistent with the fact that the HMWA recognised by 44C12 is found in the soluble fraction after hypo-osmotic treatment of the parasites (see next section).

Since the interaction of antigen and antibody in the conditions under which the ELISA is performed do not exactly mimic the interaction of antibody with the parasite *in vivo* we cannot directly extrapolate the ELISA results to the *in vivo* situation, however the lack of reactivity of 97H9, a MAb that showed high avidity for, and stability of interaction with, infective parasites in seawater adds to the proposal that in hypo-osmotic conditions the parasites shed the glycocalyx.

Therefore, the inhibition of infection assay proved not to be applicable to the assessment of MAbs 44C12, 47A1 and 48G6 that had previously been shown to block attachment of parasites in the *in vitro* assay. This was most likely due to the fact that the parasites shed the glycocalyx in the hypo-osmotic conditions that are necessary for the optimised binding of MAbs to the parasite.

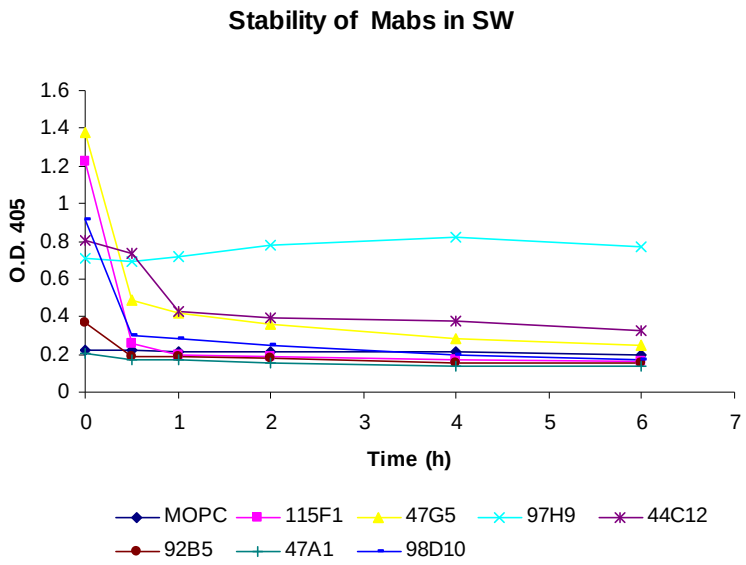


Figure 13: Stability of the interaction of MAb by ELISA using WT *Neoparamoeba* antigens. Amoeba in sea water (SW) at different time points.

5. Characterization of antigens recognised by selected MAbs

The four MAbs shown to inhibit the attachment of parasites to gill explants (44C12, 48G6, 47A1 and 48H4) are IgMs that appear to recognise carbohydrate epitopes expressed by antigens unique to WT parasites. We used MAb 44C12 to identify and characterise this antigen. We were also interested in the antigen recognised by another MAb (63C4) that initially showed positive activity in the attachment assay although these results were not confirmed in further testing. Studies addressing the characterization of these two antigens are described in this section.

Antigen recognised by MAb 44C12

Results

Flow cytometric analysis of the staining of WT parasites by 44C12 showed that the MAb reacts with two different populations of WT parasites based on light scatter properties (regions R1 and R2, top panel, Fig 14). IFAT (Fig 15), flow cytometry (Fig 14), confocal microscopy (Fig 16 and Fig 17) and TEM (Fig 18) using 44C12 indicate that the glycoprotein antigen recognised by MAb 44C12 is localised mainly on the surface and specifically on the glycocalyx of the parasite. The selective reactivity of MAb 44C12 for infective parasites was confirmed by its recognition of *Neoparamoeba* spp and *N. branchiphila* derived from gills from infected fish but not *N. branchiphila* from sediment nor *N. pemaquidensis* PAO27 (Fig 15).

Immunoblot analysis of antigen preparations from WT and PA027 parasites using MAb 44C12 demonstrated that the epitope recognised by this MAb on WT parasites is a carbohydrate as reactivity with the MAb is lost when WT antigen is treated with sodium periodate which destroys carbohydrate epitopes (Fig 19). MAb 44C12 showed no reactivity on immunoblots with antigens from cultured PA027 parasites.

Furthermore, the antigen recognised by MAb 44C12 is present in the high molecular weight fraction obtained by HPLC size exclusion chromatography of the soluble fraction from WT parasites (Fig 20). This high molecular weight antigen (HMWA) fraction constitutes approximately 19 % of the total amount of soluble protein. Immunoblot analysis of the HMWA separated under reducing conditions revealed that 44C12 recognises at least four glycoprotein bands with *M*r above 200 kDa (Fig 21 and 22). A high molecular weight fraction with similar SDS-PAGE profile but with lower MWs and a different glycosylation pattern is present in PAO27 as revealed by protein staining (Fig.20 and 22). However, these antigens do not react with MAb 44C12.

An immunoblot of WT sonicated antigen fractionated under non-denaturing conditions and probed with MAb 44C12 showed 3 bands with MW above 700 kDa (Fig 23 Panel B). These macromolecules are comprised of four subunits, all recognised by the MAb 44C12 (Fig 21). The subunits are linked by disulphide bridges (Fig 23 Panel A). These molecules are resistant to enzymatic treatment with PGNase F and O-glycosidase in the presence of neuraminidase (Fig 21), PNGase A and a mixture of O-glycosidase plus β-1,4-galactosidase, β-N-acetylglucosaminidase and neuraminidase (data not shown). In order to identify and sequence the protein core(s) of these molecules chemical deglycosylation of a semi-pure HMWA was performed using TFMS. This treatment yields several protein bands of approximate MW 46, 34, 28 and 18 kDa (Fig 24). This result indicates that these antigens are heavily glycosylated and the carbohydrates contribute at least 70% of the total mass of the high molecular weight glycoproteins. When taken together these results point to the 44C12-reactive antigens being secreted mucins present in the glycocalyx layer

that surrounds the parasite. In order to discount the possibility that these molecules are derived from the mucus of the host fish, we performed immunoblots using gill scrapings from non-infected fish acclimated in both fresh and sea water, and found that MAbs from Group 1 (i.e. those reactive with HMWA) do not react with antigens from uninfected gills while MAbs from Group 4 cross-react with fish mucus. Immunohistochemistry of infected gills probed with 44C12 showed reactivity specific to the parasites attached to the gill lamellae (Fig. 25). No reactivity with gill tissue was observed and the antibody reacted equally well with parasites present in gill tissue sections from field and laboratory infected salmon.

In order to determine if these glycoproteins were anchor to the membrane via a glycosylphosphatidylinositol (GPI) anchor we treated WT parasites with phospholipase C under different conditions and tested reactivity to 44C12 by IFAT. All the parasites retained their reactivity with 44C12 after treatment. Therefore the HMWA was not GPI anchored.

In order to explore the possible involvement of glycosaminoglycans (GAGs; see Fig 26) in the epitope recognised by MAb 44C12 and/or the HMWA fractions of infective versus non-infective parasites, glycosaminoglycans (GAGs) were identified and quantified in WT parasite HMWA using the 1,9-dimethylmethylene blue metachromatic dye binding assay (DMMB assay; Farndale 1986). The levels of GAG were determined at pH 1.5 and pH 3 to allow discrimination between sulphated (pH 1.5) and total (pH 3) GAGS. The determination of GAGs at pH 1.5 yielded 0.5 μg of GAG/μg of protein for WT HMWA and 0.18 μg of GAG/μg of protein for PAO27, while at pH 3 WT HMWA and PAO27 yielded 1.6 μg and 0.25 μg of GAG/μg of protein respectively(Table 6).

The treatment of the WT HMWA with the enzymes condroitinase, keratinase and heparitinase 1 and 2 followed by quantification of released GAGs using the DMMB assay showed that all the enzymes with the exception of keratinase resulted in partial digestion of the HMWA fraction (Table 7). However, SDS-PAGE and immunoblot analysis using MAb 44C12 didn't showed any shift in *M*r of the 4 glycoproteins recognised by the antibody, indicating that the antigens recognised do not contain GAGs.

In order to purify 44C12 antigen from the other components in the HMWA we fractionated the WT HMWA by ion exchange chromatography using a MonoQ column. Eluted fractions were pooled based on the chromatogram obtained (Fig 27) and assessed by ELISA for their reactivity with a panel of MAbs consisting of: CS-56, specific for chondroitin sulphate (GAG); 97H9 specific for fish mucus; 44C12 specific for HMWA from infective parasites; 63C4 (see next section), specific for a 6kDA component present in infective and non-infective parasites (Table 8).

Reactivity with the GAG-specific MAb CS-56 was weak across all fractions, a finding that was consistent with its reactivity with unfractionated HMWA. Further analysis of the MonoQ fractions using the DMMB assay showed that only fraction 20 contained significant levels of GAGs.
Host mucus components, assessed by reactivity with MAb 97H9, were elevated in factions 1 to 14 and greatly reduced in fractions 22 to 37.
HMWA components reacting with MAb 44C12 were found to be restricted to the latter fractions in the MonoQ separation and in particular to fractions 20 to 37, while reactivity with MAb 63C4 was detected only in the fractions 20 – 21 pool.

In an attempt to further characterise the nature of the carbohydrate epitopes expressed by components of the HMWA four MAbs were sent to the Carbohydrate Interaction, Core H of the Consortium for Functional Glycomics to be analysed using glycan array technology. Two of the MAbs were IgMs that recognised a carbohydrate epitope in the HMWA (44C12 and 47G5), one was an IgG (115F1) that recognised a carbohydrate epitope in the same fraction and the fourth MAb, used as a non-carbohydrate reactive control, was an IgM that recognised a peptide epitope. The three IgMs, including the peptide-reactive control, exhibited low level reactivity (below 5,000RFU) with transferrin and ceruloplasmin under conditions were a signal of 20,000 RFU was considered positive (Fig 28). The IgG MAb 115F1 showed no detectable level of reactivity. These results were confirmed by re-testing MAbs 44C12 and 115F1 at higher concentration in the same arrays (data not shown).

Initial attempts to obtain amino acid sequence data for the core proteins expressing the carbohydrate epitope defined by MAb 44C12, using material isolated from SDS-PAGE gels, were unsuccessful due to N-terminal blockage of the proteins for the Edman degradation (data not shown). In a further attempt to isolate and characterize the core proteins in the HMWA, acid deglycosylated HMWA was delglycosylated by treatment with TFMS and fractionated on by reverse phase chromatography to separate the core proteins. Four significant protein components were observed consistent with previous SDS-PAGE analysis of deglycoslylated material (Fig 29). Gel plugs or fractions obtained from the separations were reduced and alkylated with TBP and DTT, digested with trypsin and subjected to mass spectrometry analysis. Ionization results were analysed by Peaks Studio Auto De Novo sequencing programme. Ionization signals were generally low and none of the peptide sequences obtained matched known sequences in Swiss-Prot database searches. Only a few peptide sequences matches with sequences obtained in the other bands or picks from reverse phase (Table 9).

Discussion

The results of the immunolocalization studies clearly showed that the antigen recognised by MAb 44C12 is expressed predominantly on the surface of the parasite. At the same time, analysis of antigen preparations obtained by osmotic shock disruption of parasites revealed, unexpectedly, that the antigen recognised by 44C12 is present in the soluble fraction, as opposed to the membrane fraction. One possible explanation of this apparent contradiction is that this molecule was GPI anchored and as a result of the lysis of the parasites during antigen preparation specific enzymes were released and the GPI anchor cleaved. This possibility was discarded since phospholipase C treatment yielded negative results. Another possible explanation is that this molecule is closely associated with molecules anchored to the membrane but is not itself directly anchored to the parasite membrane. This explanation is supported by the TEM results that clearly showed the antibody bound to the glycocalyx of the amoeba, and by the results discussed in the previous section showing that washing of parasites in PBS appeared to remove the glycocalyx in which the 44C12 antigen is located.

This 44C12-defined antigen appears in the void volume of a Superdex 200 size exclusion column indicating a *M*r of >600 kDa. This result was confirmed by native gel electrophoresis (Fig 23 Panel B) that revealed at least three components above 700 kDa. These macromolecules are composed of the same four disulphide-linked subunits, all recognised by MAb 44C12 (Fig 21 and Fig 23 Panel A).

The glycoproteins in this complex are resistant to standard enzymatic deglycosylation techniques and this fact, together with the size and the high carbohydrate content, points to a secreted mucin. This and the fact that the complex is associated with the parasite membrane raised the possibility of it being host mucus. However, immunohistochemical and immunoblot analysis of mucus from gills from healthy fish showed that these molecules are not host derived. These results, in addition to the fact that 44C12 reacts with one isolate of cultured parasites (*N. branchiphila*), lead us to confidently conclude that 44C12 recognises antigens expressed by the parasite.

While a similar high molecular weight complex appears to be present in preparations from cultured PA027 parasites, the components of this complex are not recognised by MAb 44C12 and other MAbs specific for the same antigen (i.e. Group 1 MAbs). Furthermore, the components of the PA027 HMWA are of lower *M*r than those in WT HMWA, suggesting that one key difference between infective and non-infective parasites may be the glycans expressed in these macromolecules.

The role of any or all of the components of the HMWA from WT parasites in attachment of the parasite to host tissue is still not clear as the only direct evidence in this regard comes from the *in vitro* attachment assay, the variability of which prevents unequivocal interpretation. However, mucin-like molecules from Trypanozoma cruzi have shown to play a role in parasite adhesion to host cells (Hicks 2000) (Turner, 2002).

GAGs are present in the HMWA preparations from both WT and PA027 parasites but there are clear quantitative and qualitative differences. GAGs are present in lower amounts in the HMWA of PAO27 parasites. Furthermore, analysis at pH 3 which allows detection of both sulphate and carboxyl groups, and at pH 1.5 which predominantly facilitates the detection of sulphate groups, demonstrated that the GAGs in WT versus PA027 are of different composition (see Table 6). One likely explanation for this is that the composition of the GAGs in the WT parasite HMWA is heavily influenced by material of fish origin.

The possibility that GAGs constitute or significantly contribute to, the epitope recognised by MAb 44C12, thus explaining the specificity of the MAb for infective parasites, was excluded by the finding that treatment of WT HMWA with GAG specific enzymes did not alter the ability of 44C12 to recognise the characteristic antigen pattern in the preparation. While there are clearly other components in the HMWA fraction that contain GAGs they are not recognised by MAb 44C12 and similarly reacting MAbs.

Failure to detect reactivity of selected, HMWA reactive MAbs in glycan arrays indicates that the carbohydrate epitope recognised by these antibodies is not represented in the arrays.

The limited mass spectrometry data obtained on four peptides isolated by reverse phase HPLC fractionation of HMWA, and from SDS-PAGE separation of the same material, revealed some common sequences; i.e the APLLSDNYK and FLSASK sequences in the 45 to 50 kDa and 26 to 30 kDa peptides from SDS-PAGE, and the ALSGWGNTR sequence found in peaks 2 and 3 from the reverse phase column. However, there were also a significant number of sequences identified that were not shared across different components. Furthermore, none of the sequences obtained revealed homologies to any known proteins in the databases. We conclude that while the protein cores of the HMWA components recognised by MAb 44C12 may share some sequence similarities, there are also significant differences, and homologues of these proteins do not appear to have been previously identified in other species.

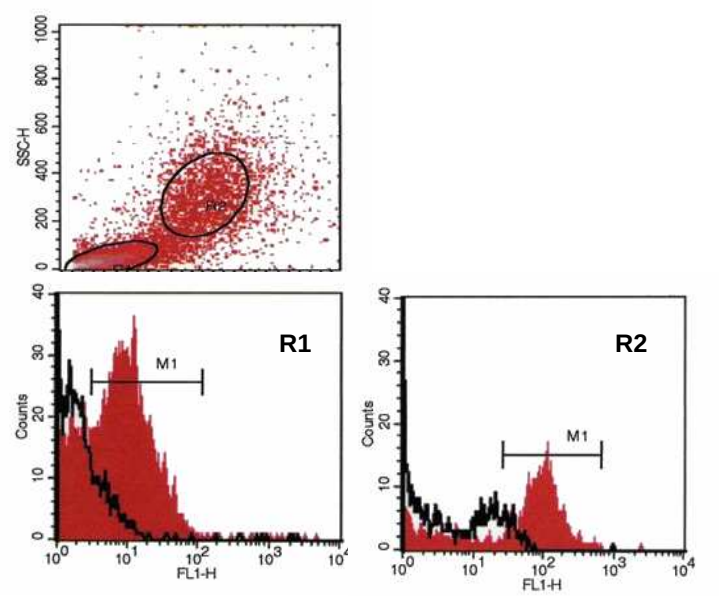


Figure 14: Flow cytometric analysis of the reactivity of MAb 44C12 with WT parasite.

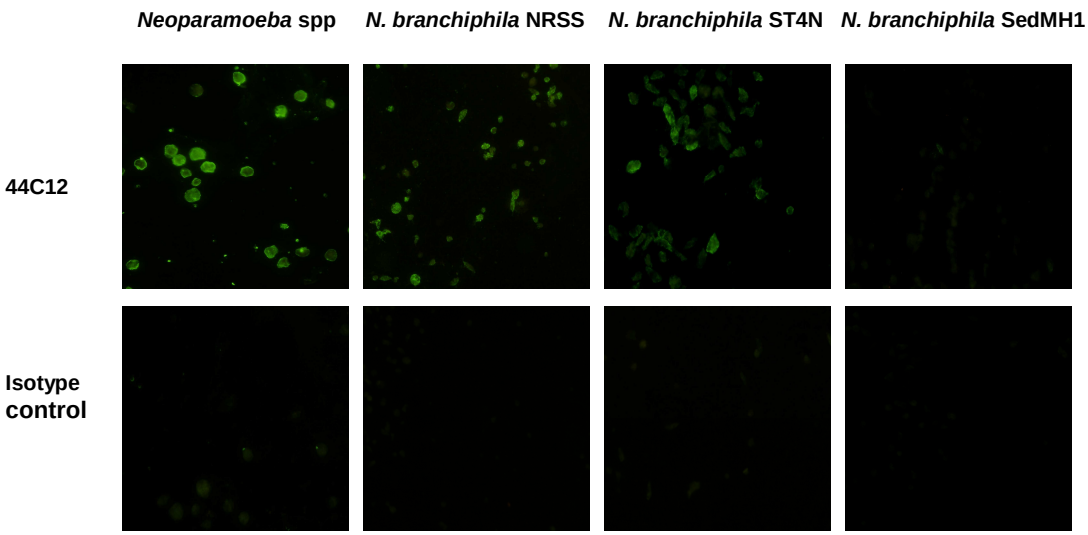


Figure 15: Analysis by IFAT of the reactivity of MAb 44C12 with WT parasites and different isolates of *Neoparamoeba branchiphila*.

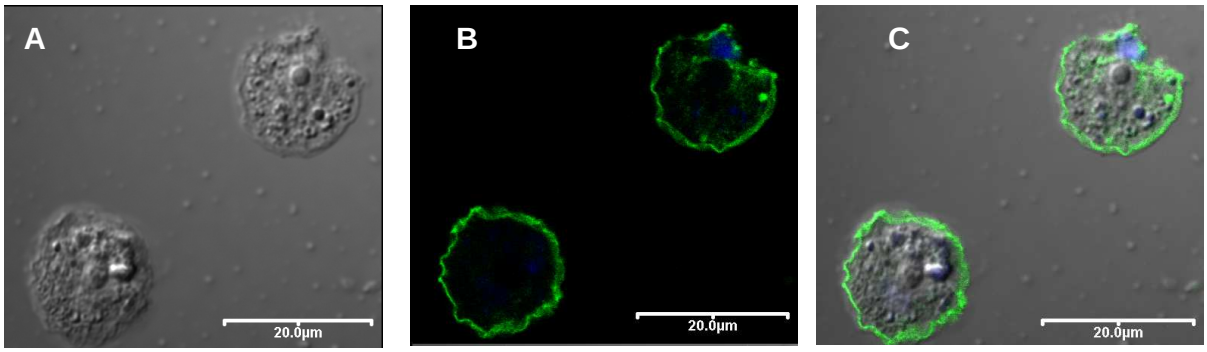


Figure 16: Immunolocalization, by confocal microscopy, of the antigen recognised by MAb 44C12. A - DIC image of WT parasites. B - Fluorescence image of a confocal internal section of the cells stained with MAb 44C12 (green) and a nuclear stain (blue). C - Images 1 and 2 superimposed.

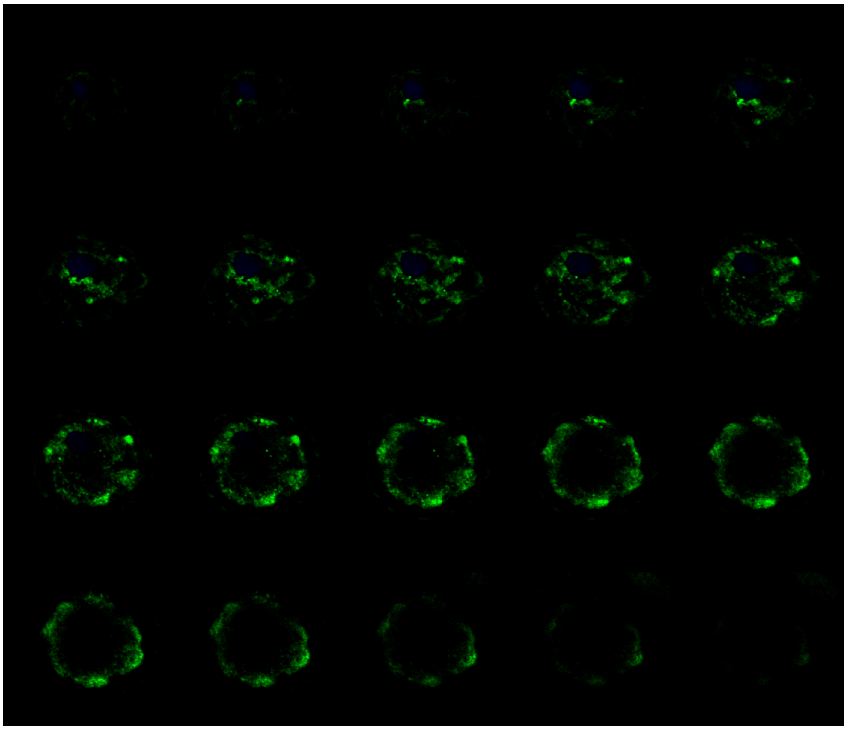


Figure 17: Fluorescence image of all the confocal microscopy internal sections of WT parasites stained with MAb 44C12.

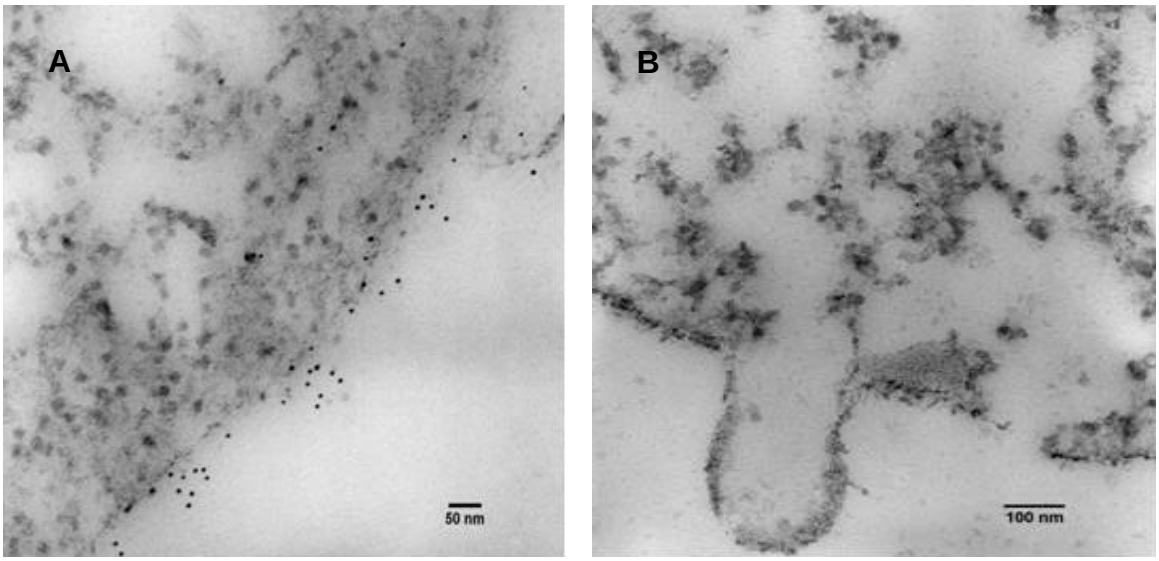


Figure 18: Immunolocalization by TEM of the HMWA on *Neoparamoeba* spp. Sections were incubated with MAb 44C12 and antibody binding detected with goat-anti-mouse IgM, 5 nm gold conjugate. Purified mouse IgM (MOPC-104E, Sigma, USA) was used as isotype control.

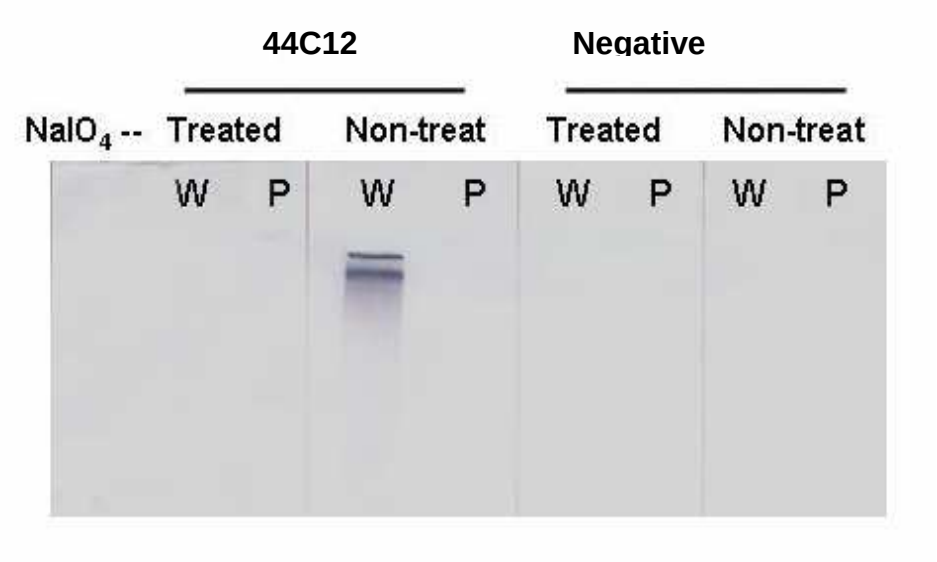


Figure 19: Immunoblot analysis of the reactivity of MAb 44C12 to WT (W) and PAO27 (P) antigens treated and untreated with NaIO4. Antigens were resolved in a 7.5 % SDS-PAGE gel.

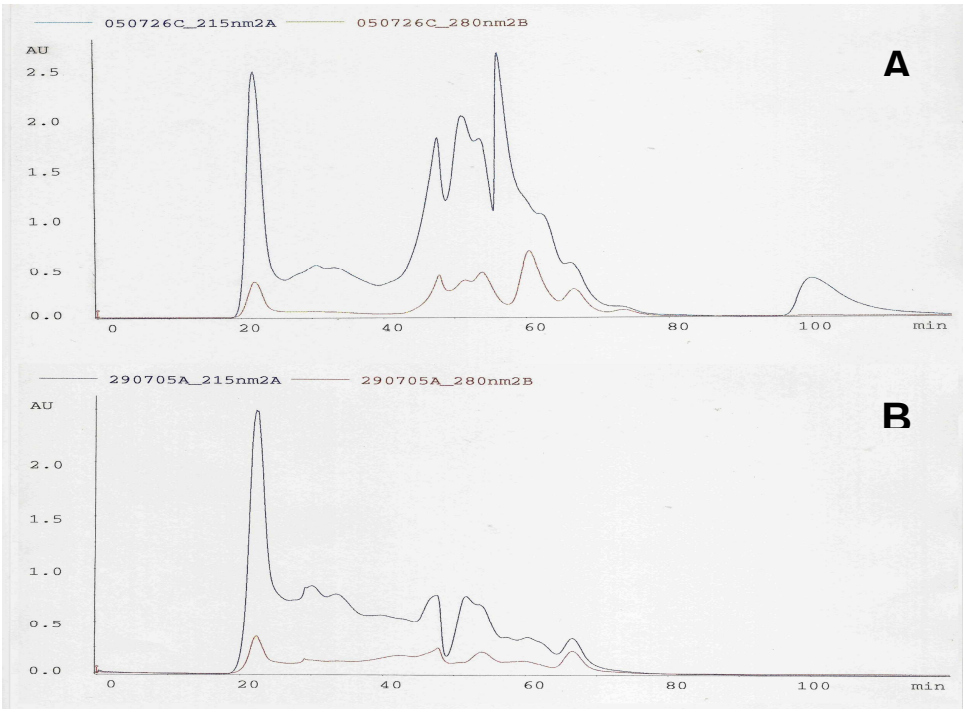


Figure 20: Fractionation of WT (Panel A) and PAO27 (Panel B) soluble fractions by size exclusion HPLC using a Superdex 200 column (Pharmacia, Sweden).

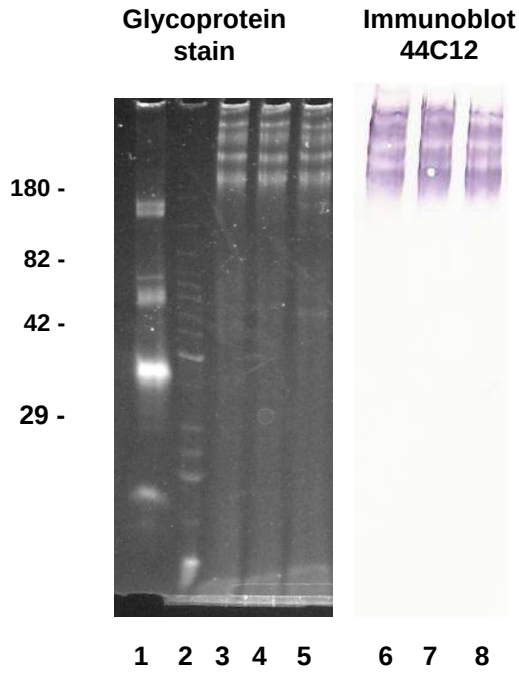


Figure 21: SDS-PAGE stained for glycoproteins and immunoblot analysis of HMWA treated with N-glycosidase (Lanes 4 & 7), treated with N and O-glycosidase and neuraminidase (Lane 5 & 8), untreated HMWA (Lane 3 & 6) and MW markers (Lanes 1 & 2). Antigens were resolved in a 4 to 15% gradient gel

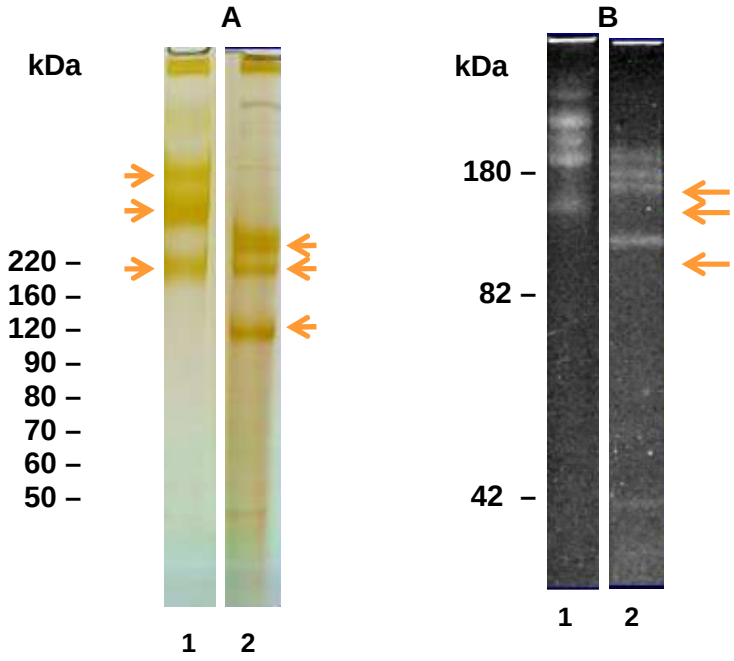


Figure 22: SDS-PAGE analysis of WT HMWA. Silver staining (Panel A) of WT HMWA (Lane 1) and PAO27 HMWA (Lane 2). Glycoprotein stain (Panel B) of WT HMWA (Lane 1), WT whole parasite antigen (Lane 2) and PAO27 whole parasite antigen (Lane 3).

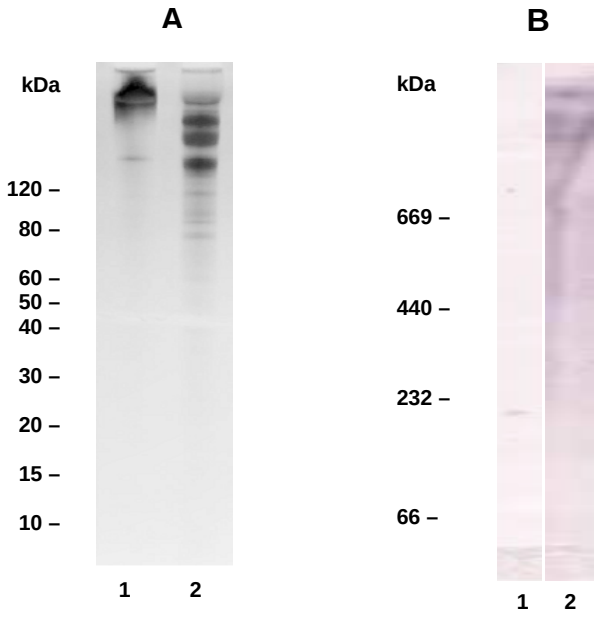


Figure 23: PAGE analysis of HMWA. Panel A – Silver stained SDS-PAGE of non-reduced WT HMWA (lane 1) and reduced WT HMWA (lane 2). Panel B - Native PAGE of WT antigen transferred to nitrocellulose and probed in lane 1 with negative control MOPC 104E (Sigma) in lane 2 with MAb 44C12.

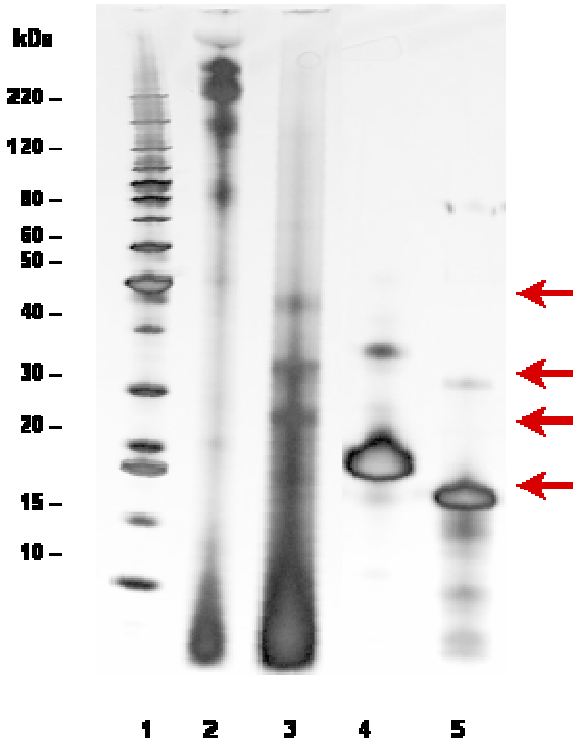


Figure 24: SDS-PAGE analysis of the HMWA from WT parasites treated and untreated with TFMS. Lane 1 - Markers. Lane 2 - Untreated WT HMWA. Lane 3 - Treated WT HMWA. Lane 4 - Untreated RNase control of deglycosilation. Lane 5 - Treated RNase control.
Antigens were resolved in a 4-12% gradient NuPAGE Novex Bis-Tris gel (Invitrogen).

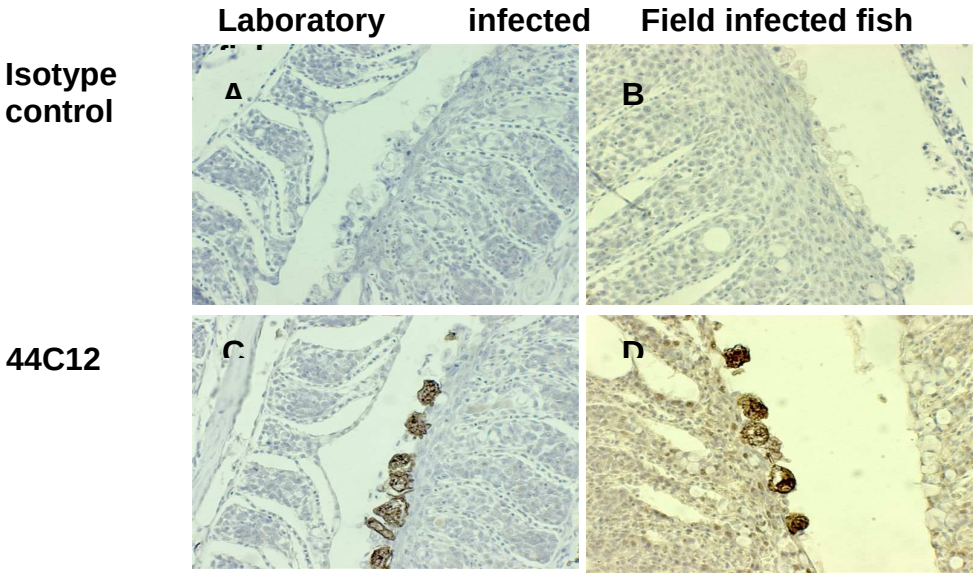


Figure 25: Immunohistochemistry analysis of gill sections from laboratory infected *Neoparamoeba* spp infected salmon (Panels A and C) and field *Neoparamoeba* spp infected salmon (Panel B and D) probed with isotype control IgM MOPC 104E (Panels A and B) and MAb 44C12 (Panels C and D).

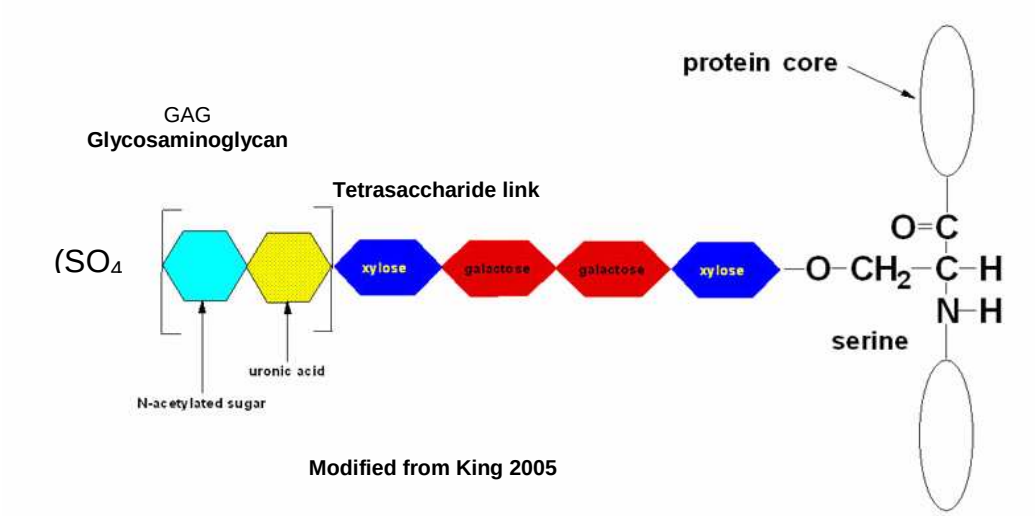


Figure 26: GAGs are long linear polysaccharides containing repeating disaccharide units. The disaccharides contain N acetyl sugars (NAcGal or NacGlc) and uronic acid (glucuronate or iduronate). They are negatively charged due to the high N and O sulfation that varies molecule to molecule. The majority of GAGs are linked to core proteins forming proteoglycans or mucopolysaccharides. The linkage of GAGs to the protein core involves a specific tetrasaccharide which is coupled to the protein core through an O-glycosidic bond. Sulphation of GAG can occur at all free NH2 or OH groups.

Table 6: Differential quantification of GAGs at pH 1.5 and pH 3.

	µg GAGs / µg protein	
	pH 1.5	**pH 3**
WT	0.5	1.6
PAO27	0.18	0.25

Table 7: Quantification of different GAGs in WT HMWA using enzymatic treatment followed by DMBM assay.

	Enzyme	WT GAGs (µg)
HMWA	----------	1.5
	Chondroitinase ABC	0.5
	Keratinase	0
	Chondroitinase ABC + Keratinase	0.6
	Heparitinase	0.4
	Heparitinase II	0.3
BNC-PG Control	----------	2.5
	Chondroitinase + Keratinase	0
Heparan sulfate Control	----------	2.6
	Heparitinase	0
	Heparitinase II	1.6

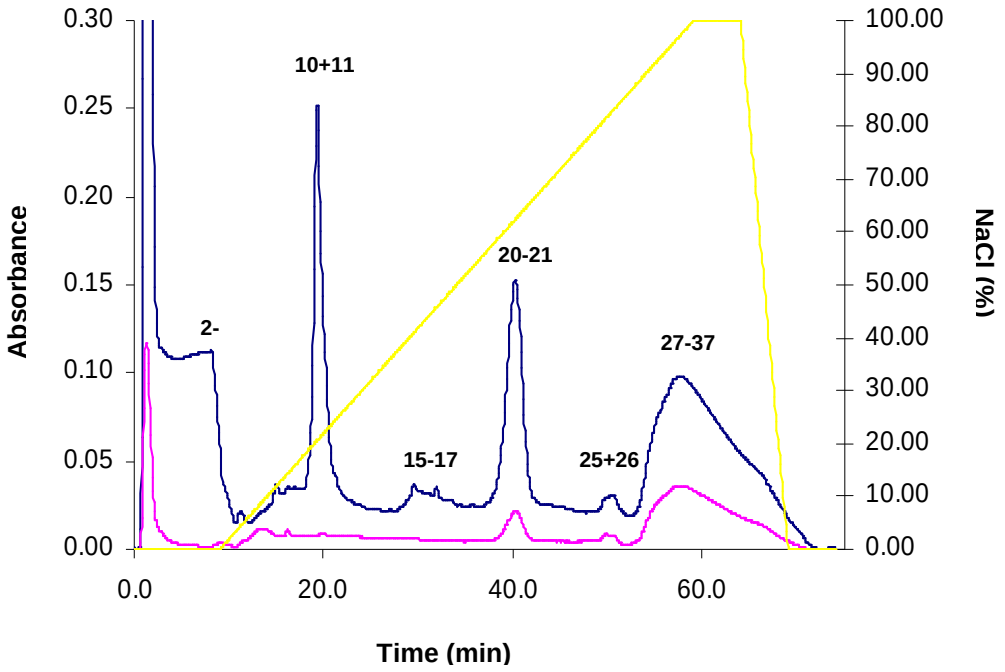


Figure 27: Anion exchange (Mono Q) HPLC fractionation of HMWA of WT *Neoparamoeba* spp. Absorbance at 220 nm is shown in blue and at 280 nm in pink. The gradient concentration of NaCl used to elute bound proteins is shown in yellow.

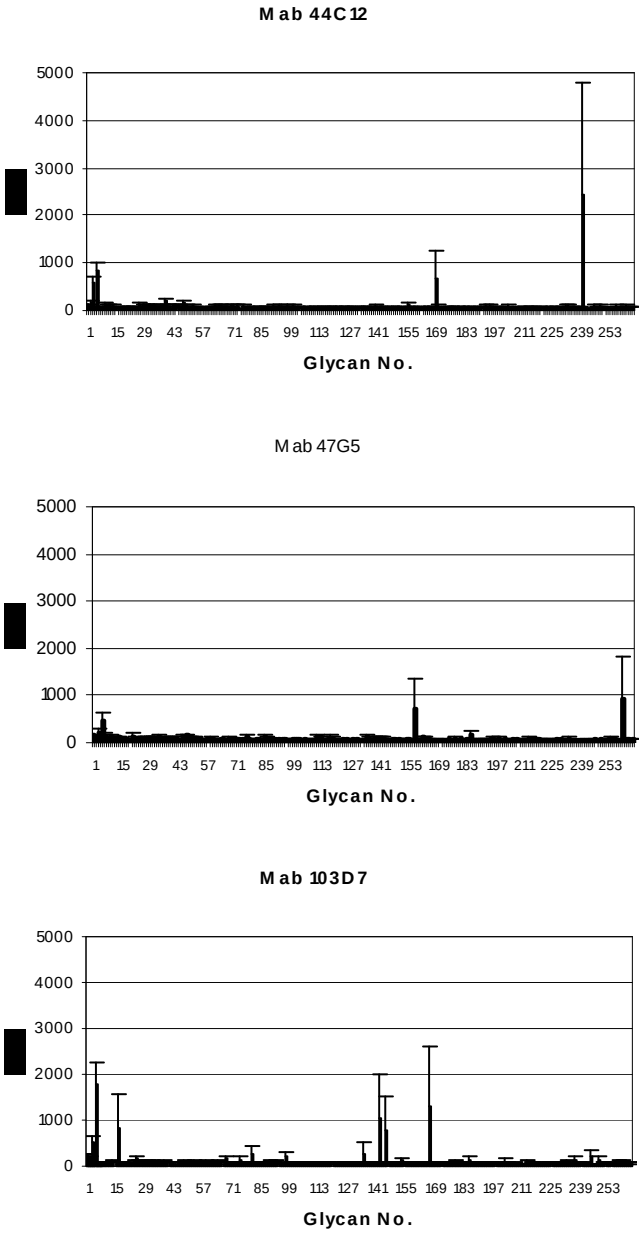


Figure 28: Glycan array analysis of the MAbs recognising the HMWA. Supernatants from the hybridomas producing the MAbs 44C12 (IgM to carbohydrate epitope), 447G5 (IgM to carbohydrate epitope), 115F1 (IgG to carbohydrate epitope) and negative control 103D7 (IgM to peptide epitope) were tested. No signal was obtained for 115F1.

Table 8: ELISA of WT fractions obtained from MonoQ separation probed with a panel of monoclonal antibodies

MAb	Unfrac	Pooled fractions										
		1	2 - 5	6 - 9	10 -11	12 -14	15 -17	18+19	20+21	22 -24	25+26	27 -37
CS-56	+/-	+/-	+/-	+/-	+/-	+/-	+/--	+/--	+/--	+/--	+/--	+/--
97H9	+++	+++	++	+++	+++	++	+	+	+	+/-	+/-	+/-
44C12	+++	-	-	-	-	-	-	+	+++	++	++	+++
63C4	++	-	-	-	-	-	-	-	+/-	-	-	-

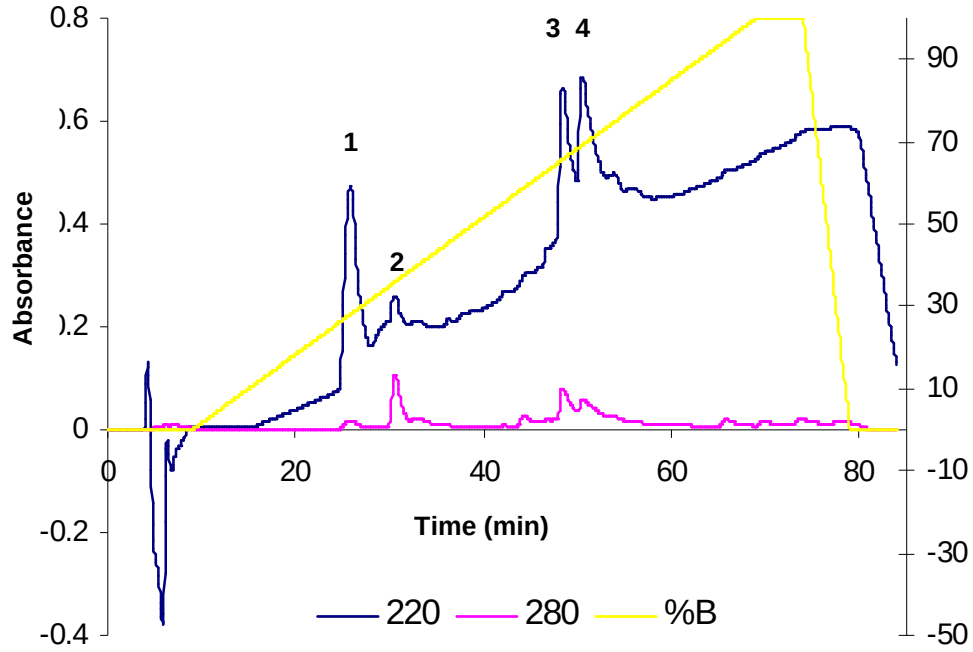


Figure 29: Reverse-phase HPLC fractionation of degycosylated HMWA. Absorbance at 220nm is shown in blue. The concentration gradient of acetonitrile used to elute bound proteins is shown in yellow.

Table 9: Mass spectrometry sequence data of components of HMWA fraction.

Sample origin	Band or peak	MS Sequence data
SDS-PAGE	45 to 50 kDa	SxxTHLR …SGYLR APLLSDNYK ……..FLSASK
SDS-PAGE	32 to 36 kDa	….ATHLR
SDS-PAGE	26 to 30 kDa	…THLR …SGYLR APLLSDNYK ……..FLSASK
SDS-PAGE	16 to 21 kDa	*nd*
Reverse phase	Peak 1	No matches
Reverse phase	Peak 2	ALSGWGNTR
Reverse phase	Peak 3	ALSGWGNTR
Reverse phase	Peak 4	No matches

Antigen recognised by MAb 63C4

Preliminary analysis of MAb 63C4 showed that it possessed inhibitory activity in the *in vitro* parasite attachment assay (data not shown) and as a result characterisation of the antigen recognised by this MAb was commenced. This work was ceased when attempts to reproduce the attachment inhibitory effect of the MAb failed due to inconsistencies in the assay as discussed earlier.

Results

MAb 63C4 recognises a carbohydrate epitope expressed in both PAO27 and WT parasites. The antigen recognised in WT parasites has a *M*r of 3-6 kDa as determined by SDS-PAGE under reducing and non-reducing conditions (Fig 30 Panel B). Immunoblot analysis with 63C4 of material fractionated by electrophoresis under native (non-denaturing) conditions showed a smear with the stronger reactivity at *M*r higher than 650 kDa (Fig 30 Panel A). This result was confirmed by ELISA where MAb 63C4 showed reactivity with the HMWA obtained by size exclusion fractionation of the WT parasite soluble fraction. Reactivity with 63C4 was also seen in a fraction obtained from the MonoQ column (Fig 27 and Table 8).

Immunolocalization of the antigen recognised by 63C4 by confocal microscopy showed a predominant expression of the antigen on the surface of WT parasites while in PAO27 parasites the antigen appeared to be predominantly localised in granules inside the cell (Fig 31).

Two unsuccessful attempts were made to obtain amino terminal sequence data for the 3-6 kDa antigen using material excised from SDS-PAGE gels, with the main problems being the presence of more than one peptide (i.e. a mixed peptide sequences) and the presence of proline residues that interrupted the sequencing process.

Discussion

Although the inhibitory activity of the MAb 63C4 in the in vitro attachment assay was not confirmed, we think that the presence of the antigen recognised by this MAb in the HMWA is relevant. Of particular interest is the fact that this antigen is expressed mostly, although not exclusively, on the surface of WT parasites while its expression is predominantly intracellular in non-infective parasites. Thus, this different pattern of expression may be significant with respect to the infectivity of *Neoparamoeba* spp. Furthermore, the 3-6 kDa glycopeptides are present in the HMWA fraction where they appear to be non-covalently associated with the higher molecular weight glycoproteins recognised by MAb 44C12.

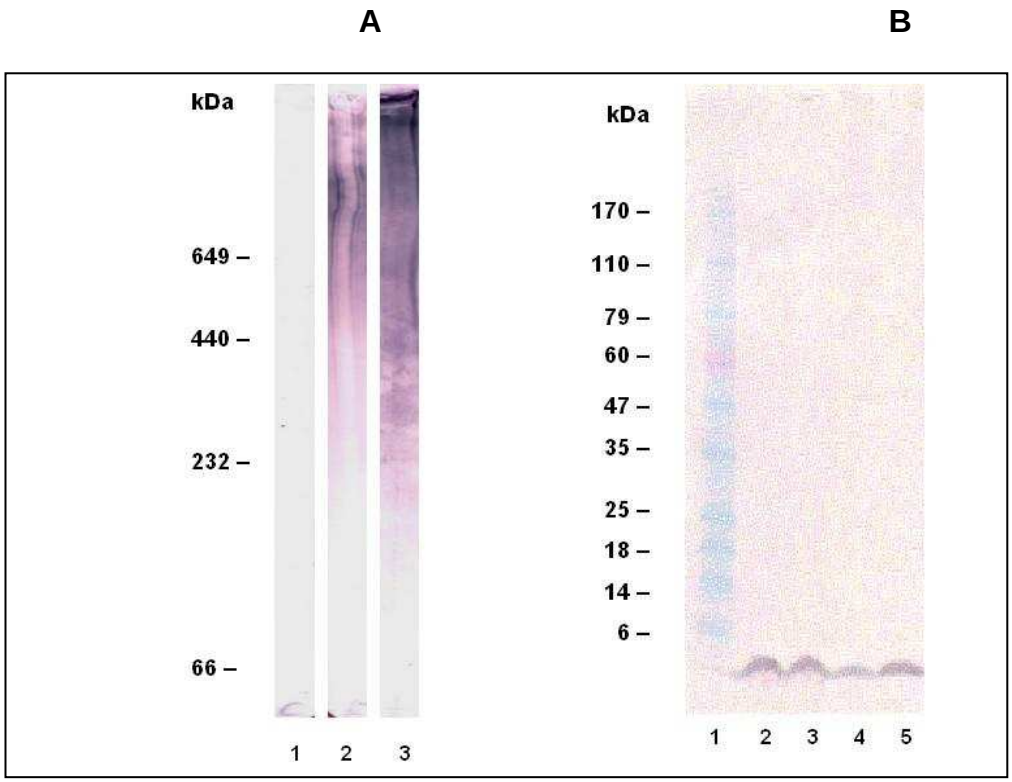


Figure 30: Immunoblot analysis of the antigen recognised by MAb 63C4. Panel A - Native PAGE. Lane 1 - MOPC 104E (Isotype control), lane 2 - MAb 44C12 and lane 3 - MAb 63C4. Panel B – SDS-PAGE. Lane 1 – Markers, lane 2 - WT Ag non-reduced, lane 3 – WT Ag reduced, lane 4 – HMWA - non-reduced, lane 5 – HMWA reduced.

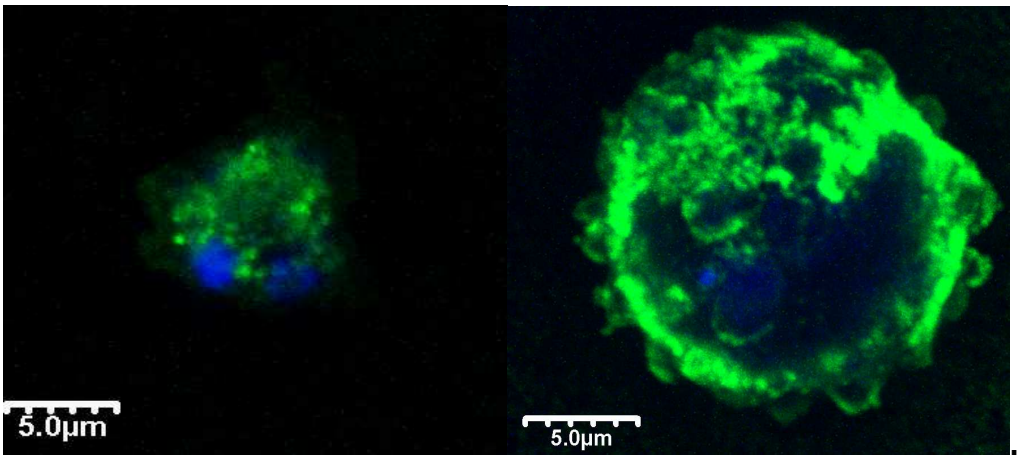


Figure 31: Immunolocalization, by confocal microscopy, of the antigen recognised by the MAb 63C4 on PAO27 parasites (left panel) and WT parasites (right panel).

6. Immunization/challenge trial with HMWA

An immunisation/challenge experiment was undertaken at University of Tasmania to analyse the immunogenicity of the HMWA in salmon and assess the potential of this preparation to protect immunised salmon from challenge with infectious *Neoparamoeba* spp.

Results

All fish immunised with HMWA (Group 1) exhibited significant antigen-specific serum antibody titres at day 49 while significant antibody levels were not detected in fish from Groups 2 and 3 (Fig 32). Specificity of the antibody response in Group 1 fish for components of the HMWA was confirmed by immunoblotting (Fig 33). Furthermore, the antibody response to infection seen in the non-immunised fish (Group 2 and 3) at day 39 post-challenge showed the same immunoblot profile as the response developed by the HMWA immunised fish (Group 1; Fig 35, lanes 12 and 14).

In contrast to the response detected in serum, specific antibodies were not detected in the mucus of any fish at day 49. However, a low but detectable level of antibody was present in the mucus of the 5 Group 1 fish that survived to day 105 of the experiment (i.e. 39 days post parasite challenge; Fig 34).

Some non-AGD mortalities were noted in all groups due to an unrelated skin infection prior to challenge with infectious parasites. Thus, at time of challenge, Group 1 consisted of 10 fish, Group 2 11 fish and Group 3 14 fish (Table 10). Analysis of the survival of fish in these 3 groups following challenge with infective parasites showed that despite the presence of specific serum antibody, fish immunised with HMWA were not protected from infection with the parasite (Table 10 and Figure 35). Surprisingly, fish in the adjuvant control group (Group 2) exhibited significantly lower mortality (0%) compared to both the antigen immunised group (Group 1; 50%) and fish in Group 3 that were injected only with saline (43%). The statistical significance of these results was confirmed by CSIRO Mathematical and Information Services whose report (Appendix 3) identifies significant differences between all the treatment groups.

Gill histology was performed by Dr Phil Crosbie, University of Tasmania, on all fish that survived to day 105 of the trial (i.e. 39 days post challenge). Evidence of AGD, in the form of significant levels of gill lesions, was observed in all surviving fish (Table 11).

Discussion

Immunization of salmon with the whole HMWA fraction derived from infective *Neoparamoeba* spp failed to protect fish from subsequent challenge with the parasites despite the presence of a significant HMWA-specific serum antibody response. However, the lack of protection of the immunised fish could be explained by the failure to elicit an appropriate immune response at the gill. Although the fish immunised with HMWA developed a strong systemic antibody response, the level of antibody detected in gill mucus was very poor and arguably not effective with respect to protecting against AGD. Better immunization regimes need to be developed to elicit a strong immune response at the gills.

The observation that fish immunised with adjuvant alone exhibited a survival advantage over fish immunised with HMWA plus adjuvant suggests that the HMWA may contain “immunosuppressive” components that can inhibit the protective response resulting from the administration of adjuvant alone. These molecules could be part of the strategy of the parasite to avoid the immune response and therefore may be responsible for the lack of effective protection from re-infection with *Neoparamoeba* spp of salmon that have recovered from AGD.

With only these results we cannot discard the potential protective value of the antigen recognised by 44C12 since the fraction used for immunization was a complex mixture purified only on the basis of molecular weight. These results do suggest that the HMWA fraction contains “immunosuppressive” molecule/s that are potent enough to block the protective response developed by immunisation with FCA alone. However, the fact that fish immunised with HMWA developed strong antigen-specific serum antibody indicates that the “immunosuppressive” effect does not impact on the humoral antibody response.

The fact that all surviving fish showed significant levels of gill lesions leaves two possibilities with respect to the apparent protective effect of adjuvant alone (Group 2). Firstly, the effect may be short lived, and hence the fish in this group would have shortly succumbed to the disease. Alternatively, the protective effect may be independent of gill lesion formation and may act at an as yet unknown physiological level. The latter is supported by the results obtained by Vincent *et. al.* (2006) that found that fish resistant to AGD infection due to previous exposure showed similar gill pathology to naïve fish when challenged with infective parasites

This trial clearly demonstrates that i.p. immunization with this HMWA fraction does not elicit a protective effect in naïve salmon despite the production of significant levels of specific serum antibody.

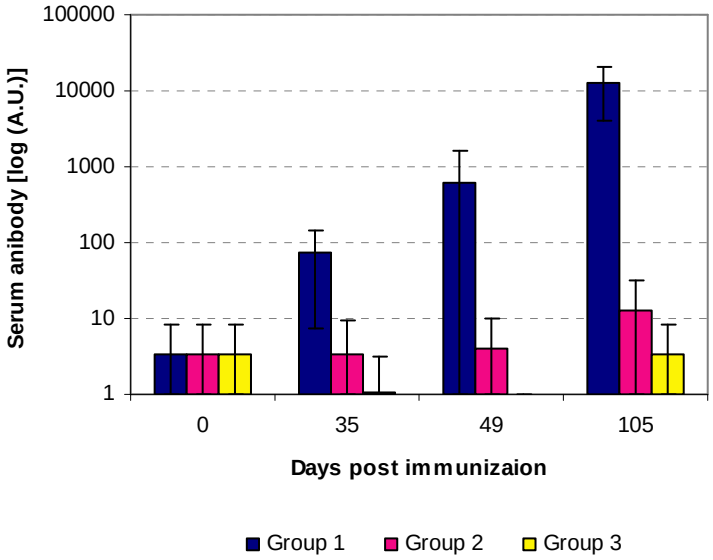


Figure 32: Serum anti-HMWA response in fish immunised with HMWA emulsified in FCA and boosted at day 35 with HMWA in FIA (Group 1), fish immunised with PBS emulsified in FCA and boosted at day 35 with PBS in FIA (Group 2), and fish injected with PBS alone (Group 3). Fish were challenged with *Neoparamoeba* spp at day 66.

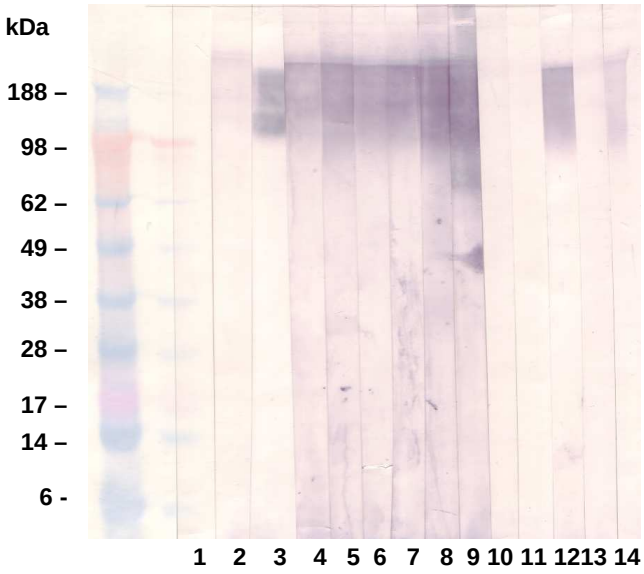


Figure 33: Western blot analysis of representative serum samples from fish immunised with HMWA and challenge with *Neoparamoeba* spp. HMWA was separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with serum from individual fish in the immunisation trial. Binding of fish antibody was detected with an anti-salmonid immunoglobulin-Alkaline Phosphatase conjugate (5F12) (Immuno-Precise). Individual lanes on the gel were probed with: 1- PBS-BSA; 2- Day 0 serum; 3 - 44C12D10 MAb; 4 - Day 35 Group 1; 5 - Day 35 Group 1; 6 - Day 49 Group 1; 7 - Day 49 Group 1; 8 - Day 105 Group 1; 9 - Day 105 Group 1; 10 - Day 49 Group 2; 11 - Day 105 Group 2 ; 12 - Day 105 Group 2; 13 - Day 49 Group 3; 14 - Day 105 Group 3.

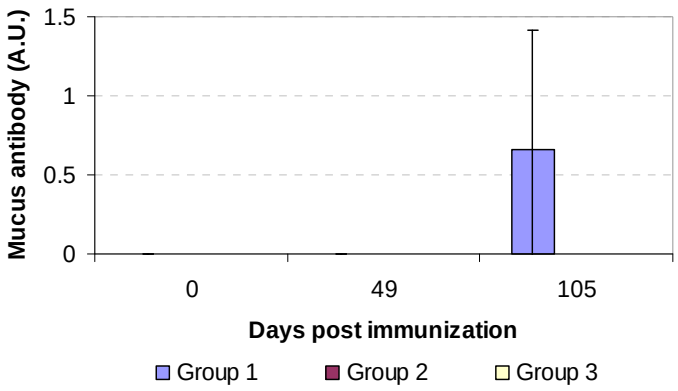


Figure 34: Mucus anti-HMWA antibody response in fish immunised with HMWA emulsified in FCA and boosted at day 35 with HMWA in FIA (Group 1), fish immunised with PBS emulsified in FCA and boosted at day 35 with PBS in FIA (Group 2), and fish injected with PBS alone (Group 3). Fish were challenged with *Neoparamoeba* spp at day 66.

Table 10: Survival of fish following challenge with infectious parasites

Group	No. surviving fish (day 0 post challenge)	No. surviving fish (day 39 post challenge)
1	10	5
2	11	11
3	14	8

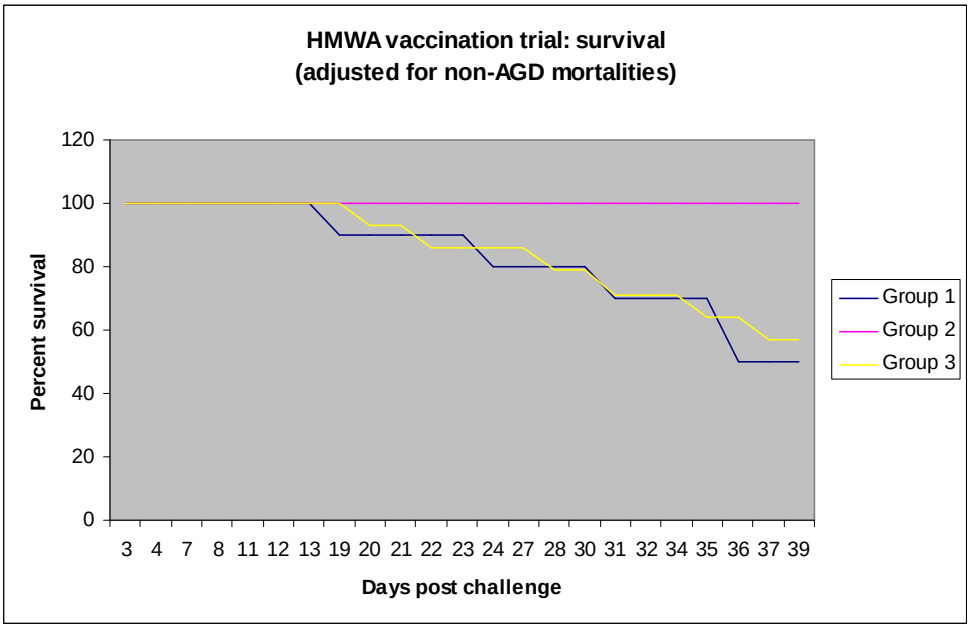


Figure 35: Survival of the immunised fish following challenge with *Neoparamoeba* spp. Group 1 - immunised with HMWA emulsified in FCA and boosted at day 35 with HMWA in FIA. Group 2 - immunised with PBS emulsified in FCA and boosted at day 35 with PBS in FIA. Group 3 - fish injected with PBS alone. Fish were challenged with *Neoparamoeba* spp at day 66.

Table 11: Histological confirmation of AGD in surviving fish.

Group	No. of fish examined	% gill lesions	
		Range	Mean +/- SD
1	5	59-93	73 +/- 13
2	11	41-94	76 +/- 17
3	8	17-100	76.5 +/- 27
	7*	65-100	85 +/- 14

*** excludes low value outlier**

7. Immunization/challenge trial with Freund's Adjuvant

Given the unexpected finding that administration of Freund's Complete Adjuvant (FCA) in the absence of parasite antigen resulted in significant protection from mortality due to AGD (Fig 35) an additional trial was undertaken to confirm the effect of adjuvant alone. Fish were primed on day 0 and boosted on day 35 with either: FCA followed by Freund's Incomplete Adjuvant (FIA) (Group 1), FIA and FIA (Group 3) or PBS only (Group 2). For ethical reasons it was not possible to administer FCA at both priming and boosting stages due to the potential inflammatory response that such a regime might induce. Fish were challenged with infective parasites on day 66, and AGD related mortalities were recorded up to the termination of the experiment on day 136 (68 days post challenge) when all surviving fish were sacrificed and assessed for AGD infection by examination of gill histology.

Result

The mortalities registered for Group 1 (primed with FCA and boosted with FIA) and Group 2 (infection control) at day 39 post challenge were very similar (18% and 16% respectively) while the mortality level for Group 3 (primed and boosted with FIA)was slightly lower (10%) (Table 12). At the termination of the experiment on day 134 the untreated infection control group (Group 2) registered mortalities of 70% while fish in Group 1 showed an overall mortality of 61% and fish receiving FIA exhibited a higher rate of survival with a mortality level of 46%. Despite the apparent improved survival rate in the FIA group, statistical analysis of the data using the Kaplan-Meier Survivor Function and Cox's Proportional Hazard's Model failed to identify any significant differences between the treatment groups ($P = 0.667$).
Gill histology was performed at the end of the experiment on all surviving fish. Evidence of AGD, in the form of significant levels of gill lesions, was observed in all surviving fish (Table 13). Statistical analysis of the % of gill lesions was performed using Kruskal-Wallis Non-parametric test. No significant differences were found in the gill lesion scores between the experimental groups.

Discussion

In contrast to the earlier trial that showed 0% mortality at day 39 post challenge in the FCA control group, in this experiment fish primed with FCA (Group 1) showed no difference in mortalities compared to the untreated infection control group at day 39. However, the level of mortalities at day 39 post challenge showed that the kinetics of the *Neoparamoeba* infections were very different. In the initial trial (Fig 35) 43% of fish in the control group had succumbed to AGD by day 39 post challenge while in the second experiment (Fig 36) at day 39 only 18% mortality was recorded in the control group (Table 12). Such differences in the progression of the disease in the laboratory infection system are to be expected and could be affected by a number of parameters, including the general condition of the fish at the commencement of the experiment. In this regard, it should be noted that in the first experiment a number of fish succumbed to a skin infection (non-AGD related) prior to, and immediately after, challenge with the parasite.

The mechanisms of action of Freund's adjuvant are still a matter of debate, but it is known that both FIA and FCA produce local inflammation that attracts phagocytes and lymphocytes. The mycobacterium in FCA activates dendritic cells and macrophages and enhances co-stimulatory signals (Billiau, 2001). Therefore the protective effect observed in the fish in the initial trial immunised with Freund's

adjuvant is most probably the result of an effect on monocytes/macrophages. The different results obtained in the two trials using adjuvants could be explained by the fact that in the first trial some fish in the tank were infected with a skin pathogen. This co-infection possibly increased the recruitment of activated monocytes/macrophages (systemically activated by the adjuvant) to mucosal sites; a recruitment that otherwise would be very poor since the parasite itself doesn't induce a significant inflammatory response at the gill epithelium.
However, the level of protection obtained in the first vaccine trial, whether due to the adjuvant or a coincidental skin infection, raises interesting questions about the potential role of innate immune mechanisms in protection of salmon from AGD.

Table 12: Effect of adjuvant on the survival of salmon challenged with AGD

Group/treatment	Number of fish challenged	% AGD related mortality 39 days post challenge	% AGD related mortality 68 days post challenge
1. FCA/FIA	**38**	**18**	**61**
2. PBS/PBS	**37**	**16**	**70**
4. FIA/FIA	**39**	**10**	**46**

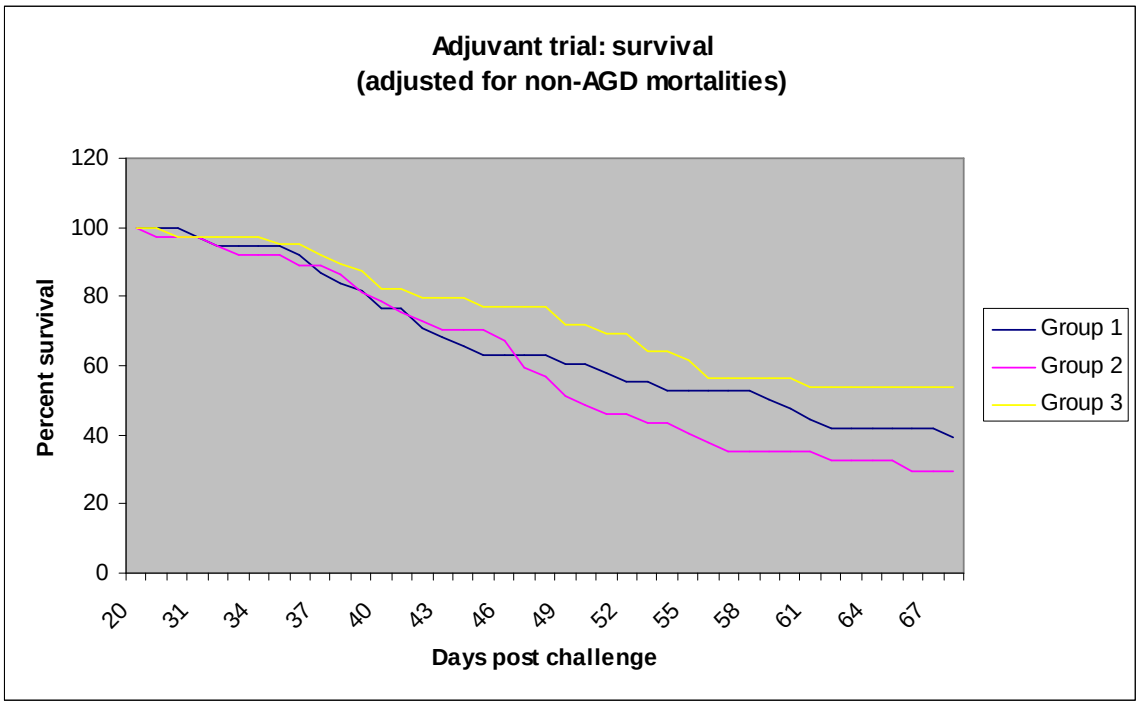


Fig. 36 – Survival of the immunised fish following challenge with *Neoparamoeba* spp. Group 1 – primed with FCA and boosted at day 35 with FIA. Group 2 – Primed and boosted at day 35 with PBS alone. Group 3 – Primed and boosted at day 35 with FIA.

Table 13 – Histological confirmation of AGD in surviving fish.

Group	No. of fish examined	% gill lesions	
		Range	Mean +/- SD
1	15	14.8-100	56±32
2	12	2.1-100	45±38
3	21	1.5-100	56±33

8. Comparison of route of immunization in the induction of a mucosal immune response.

To prevent attachment of the parasite to the gill epithelium of the host there is a clear need to induce an effective immune response at the mucosal surface of the gills. In order to maximise the response at the gills we compared different immunization regimes to select the one that elicited the best immune response at mucosal sites. Previous studies performed at UTS demonstrated that a significant antibody response could be detected in mucus from systemically (i.p.) immunised trout boosted via a mucosal surface (Cain, 2000.) In order to confirm these results in salmon we performed two experiments to compare combinations of priming/boosting immunisations via systemic and mucosal routes. Systemic immunisation was via the i.p. route while mucosal administration of antigen was via spraying of the gill surface. The following combinations were assessed to determine which would be most useful in future vaccine trials; i.p. /i.p., i.p. / spray and spray/spray.

Experiment 1

Four groups of salmon were immunised with PAO27 soluble antigen. Group 1 was primed and boosted at day 49 by i.p. inoculation. Group 2 was primed by i.p. inoculation and boosted at day 49 by spray immunisation at the gills. Group 3 was primed and boosted by spray immunisation at the gills. Group 4, the sham immunised control, was given PBS alone via the i.p. (priming) and spray (boosting) routes.

Results

Seventy percent of the fish in this experiment died due to a failure in the aeration pump servicing the aquarium. The number of fish remaining (2 from group 1, 3 from group 2, 1 from group 3 and 2 from group 4) were not sufficient to yield statistically significant results but the samples obtained from the few surviving fish were useful for the development of an ELISA to detect specific antibodies in serum and mucus samples from salmon.

Three antibody-enzyme conjugates were tested for detection of salmon immunoglobulin: mouse anti-salmonid immunoglobulin (monoclonal antibody 5F12) alkaline phosphatase conjugated (Immuno-Precise); two polyclonal rabbit anti-salmonid immunoglobulin alkaline phosphatase conjugates from Serotec and GroPep. The two polyclonal reagents showed high background reactivity particularly with the mucus samples and therefore were not used in subsequent assays.

Having established the assay individual serum and mucus samples were analysed by ELISA against PA027 antigen. Interestingly, the serum antibody responses of 2 out of 3 fish from the i.p. / spray group were higher than the response of the i.p. /i.p. group (Fig 37) with antibody levels reaching maximum levels around day 30 for the primary response and day 80 for the secondary response. The fish primed and boosted by spray alone did not exhibit significant antibody levels.

The skin mucus antibody response was positive for 2 of 3 fish from the i.p./spray group, 1 of 2 fish from the i.p. /i.p. group with the 2 fish from the i.p./spray group again being the best responders. Responding fish exhibited a peak skin mucus response at day 29 after the i.p. inoculation (priming) and all responding fish showed increased skin mucus antibody levels after boosting at day 80. The antibody response in gill mucus followed the same trend as the antibody response in skin

mucus showing a peak on day 29-post primary immunisation and an increase in the antibody levels after boosting at day 80. There was no apparent correlation in the antibody responses observed in mucus and serum for individual fish; i.e. a high serum responder did not necessarily exhibit a significant antibody response in the mucus.

The serum and mucus samples were also tested by immunoblot to confirm the results obtained by ELISA (Fig 38 and 39). All samples shown to be antibody-positive by ELISA showed positive immunoblot profiles while all antibody-negative samples failed to bind to sonicated PAO27 antigens.

The immunoblot analysis also showed that although both the systemic and the mucosal immune responses are specific to high molecular weight molecules the profiles are different (Fig 38 Panel C and Fig 39 Panel B) confirming that the antibodies detected in mucus are not derived directly from serum.

Some of these samples were also tested against WT sonicated antigen by ELISA and the results showed that when compared to negative controls the samples giving high absorbance values in ELISA using PAO27 antigens also yielded positive results against WT antigen, although at significantly lower levels (data not shown)

Discussion

The numbers of fish were not sufficient for statistical analysis but the data obtained from the few surviving fish were useful as preliminary results to design the follow up experiment and for the development of an ELISA to detect specific antibodies in serum and mucus samples from salmon. The ELISA developed and tested in this experiment was useful for the detection of serum and mucosal antibody responses to WT antigens in salmon.

The antibody response in gill mucus followed the same trend as the antibody response in skin mucus. There was no apparent correlation in the antibody responses observed in mucus and serum. Although the results were preliminary because of the loss of fish referred to earlier, the combination of primary immunisation via the systemic route followed by boosting at the gill mucosal surface (i.p. /spray immunization regime) appeared to elicit a better mucosal and systemic immune response compared to immunisation via the systemic route (i.p. /i.p.) alone. In order to confirm this tentative finding the experiment was repeated as described in page 65.

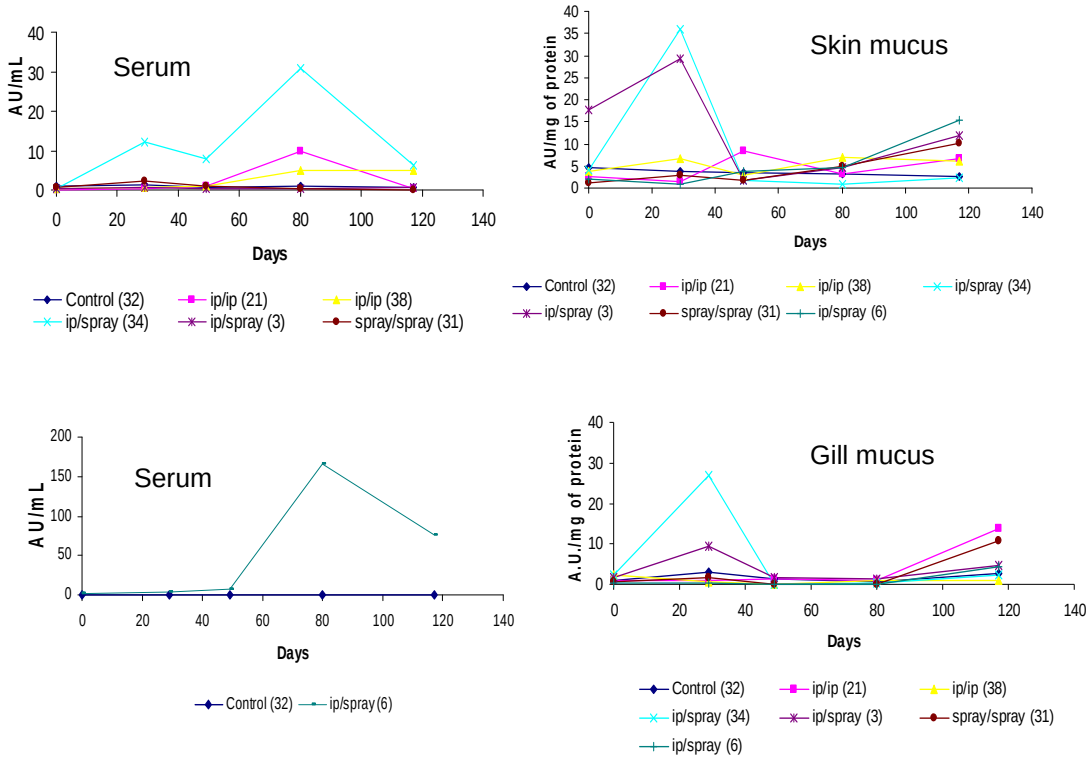


Figure 37: Antibody response, detected by ELISA, in serum and mucus samples of salmon immunised with sonicated PA027 antigen. Individual fish numbers are indicated in brackets. The serum antibody response for fish 6 is shown separately in the lower left panel as it represented a high responder with a titre of 1/28000.

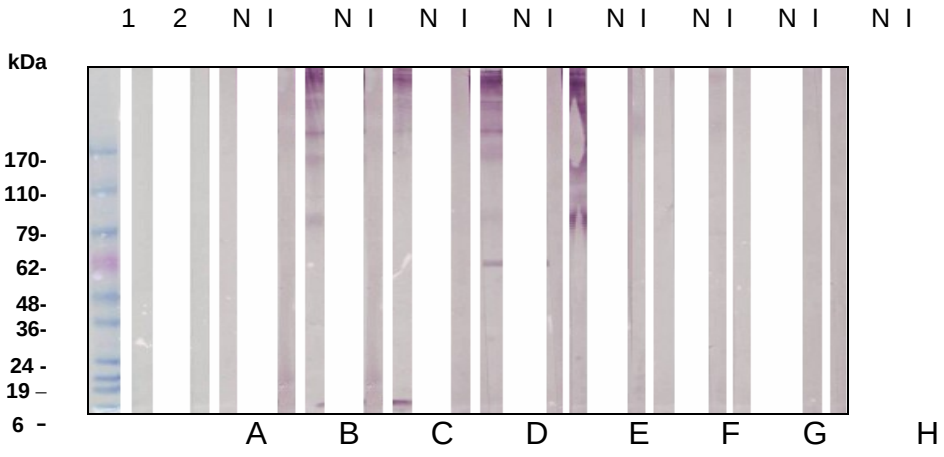


Figure 38: Immunoblot profiles of sera from fish immunised with PAO27 antigen. 10 µg of PA027 antigen was resolved on 4-20% gradient SDS-PAGE gel and transferred onto nitrocellulose. N - non-immune serum (day 0). I - serum from day 80 post immunisation. Lane 1 - Benchmark™ prestained protein ladder Lane 2 - secondary antibody control. Panels A, B, C - i.p./spray group fish 3, 6 and 34 respectively. Panel D, E – i.p. /i.p. group, fish 21, 38 respectively. Panel F and G - control group – fish 12 and 32. Panel H - spray /spray group fish 31.

Experiment 2

In the first immunisation experiment we showed that the gill mucus antibody response correlated well with skin mucus antibody levels so in the second experiment described here we only measured the skin mucus response. Furthermore, since no response was detected in the spray/spray group from the first experiment this group was eliminated in the second experiment. Therefore, only three groups of salmon were immunised with PAO27 soluble antigen in the present experiment. Group 1 was primed and boosted at day 49 by i.p. inoculation. Group 2 was primed by i.p. inoculation and boosted at day 49 by spray immunisation at the gills. Group 3, the sham immunised control, was given PBS via the i.p. (priming) and spray (boosting) routes.

Results

Serum and mucus samples were analysed by ELISA and immunoblot. Both immunised groups showed a significant systemic antibody response compared to the sham immunised control but the standard deviations of the individual responses were very high and as a result the profiles of the response cannot be considered conclusive.

No specific antibodies were detected in mucus at week 4. After boosting at week 4, the PAO27 specific antibody response for the i.p. /i.p. immunised group increased showing a peak at week 8 increasing again to reach a maximum level at day 12 or beyond. The fish boosted by spray immunization also showed a peak response at week 8, although at lower levels than Group 1. This response showed a slow but consistent decrease from week 10 to the end of the experiment (Fig 39).

After boosting at week 4 no specific antibody response in mucus was detected for Group 2 (i.p./spray immunised fish) and only two fish responded in the i.p. /i.p. immunised group.

Discussion

The results obtained in this experiment are not consistent with our previous results (Experiment 1). In this experiment the spray immunization appears to have failed to have any effect on the systemic or mucosal antibody responses of the fish in Group 2. The profile of the response in this group is consistent with i.p. priming alone. The differences in timing between priming and boosting could explain some differences but not the complete lack of response to the booster seen in Group 2.

A possible explanation is that PAO27 parasites are cultured using *E. coli* to feed the parasites. It is possible that *E. coli* molecules in the antigen preparation may have an adjuvant effect at the gills and as a consequence differences in the *E. coli* content in the antigen preparation could explain differences in the mucosal immune responses. Other possible factors include the size and/or age of the fish immunised. In our previous experiment we used fish between 300g to 500 g while the fish used in this last experiment were between 100 to 200 g. Furthermore, since we are working with outbred fish high levels of variability in antibody responses within a group (and hence large standard deviations) can be expected due to variability between individual fish.

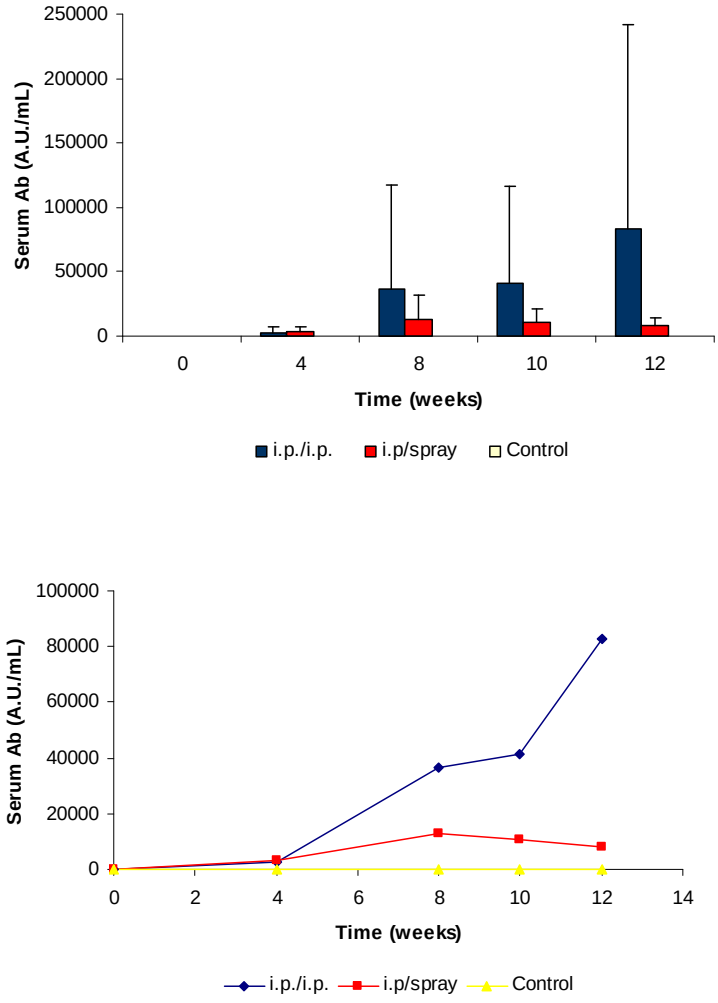


Figure 39: Antibody response, detected by ELISA, in serum samples of salmon immunised with sonicated PA027 antigen. Group 1 (blue) - primed and boosted by i.p. inoculation. Group 2 (red) – i.p. primed and boosted by spray immunization. Group 3 (yellow) – i.p. primed with PBS and boosted with PBS by spray immunization.

9. General discussion

The high number of MAbs that recognise carbohydrate epitopes unique to WT parasites is a strong indication that the main difference between infectious and non-infectious *Neoparamoeba* lies in the glycans present in the glycocalyx of infectious parasites. These MAbs were obtained from 11 fusions using a variety of antigens and immunization regimes, thus ruling out the possibility that the restricted response to carbohydrate epitopes was a reflection of one particular immunisation regime or antigen preparation. Furthermore, the HMWA from WT parasites is recognised by the majority of the MAbs specific to infectious amoeba indicating that this fraction contains the majority of the antigens differentially expressed between infectious and non-infectious *Neoparamoeba,* including the antigen recognised by MAb 44C12 and other MAbs that exhibited inhibitory activity in the *in vitro* attachment assay. Therefore we can conclude that the glycans or glycoproteins involved in the attachment of the parasite to the gill epithelium are likely to be present in the HMWA fraction.

The high level of O-glycosylation, the high molecular weight of these glycoproteins and the fact that they are not anchored to the membrane is consistent with these molecules being secreted mucins (Hicks, 2000). From the HMWA immunisation trial we have evidence that at least one or more of the components of the HMWA fraction possesses some kind of “immunosuppressive” activity. This “immunosuppressive” effect raises the possibility that these “mucins” are being secreted by the parasites to avoid or regulate the immune response of the fish. We postulate that when these molecules are secreted by the parasite they associate with molecules anchored to the surface of the parasite by ionic interactions forming a glycocalyx or protective “coat”. This “coat” is what the parasite shows to the immune system of the host and it can be shed if the conditions change, like, for example, during *in vitro* culturing of the parasites. This hypothesis would explain the dramatic changes in the surface antigens seen during culture of WT *Neoparamoeba* (Villavedra, 2005). However, an alternate explanation for this phenomenon is the possible overgrowth of *N. perurans,* the species currently considered the aetiological agent of AGD, by non-infective *Neoparamoeba*

Complex glycoproteins, particularly mucins, play a crucial role in the interaction between protozoan parasites and their hosts. For example some of these glycoproteins are recognised by dendritic cells and/or macrophages and as a consequence can exert positive or negative regulatory effects on the immune response of the host against the parasite (Hicks, 2000; Kwame Nyame, 2004). Other mucins are involved in the attachment of parasites to the host cells (Hicks, 2000; Turner, 2002).

Since the HMWA includes all the high MW molecules from the soluble fraction of the parasite these “immunosuppressive” molecules may not necessarily be localised to the surface of the parasite. However, the majority of the components identified in the HMWA (4 glycoproteins, GAGs, antigen recognised by 63C4 and fish mucus) are localised on the surface of the parasites and the size of the molecules in this fraction (>600kDa) is consistent with this finding. The secretion or shedding of molecules to modulate the immune response is a well known mechanism used by parasites to evade or modulate the immune responses of the host (Hicks 2000; Loukas, 2000; Zambrano-Villa, 2002). The hypothesis that these molecules are being secreted or shed by infective *Neoparamoeba* spp to immunosuppress the host is supported by results obtained by Young et al. (2008) on the analysis of the transcriptome of “normal” gill tissue compare to infected gill tissue. They found down regulation of

genes involved in antigen presentation and development of an adaptive immune response. Gross *et al.* (2005) also found that basal respiratory burst activity and phorbol myristate acetate-stimulated activity of anterior kidney macrophages were suppressed during *Neoparamoeba* spp infection and suggested that the parasite somehow blocks macrophage activity. We hypothesise that one or more components of the secreted HMWA from infective *Neoparamoeba* may be responsible for this effect. This should be tested by assessing the effect of different fractions or components of the HMWA on salmon macrophages and/or analysis of the transcriptome of fish immunised at mucosal sites with HMWA, and of fish infected with AGD.

As discussed earlier the protective effect observed in fish immunised with FCA was probably due to a combination of FCA-induced macrophage activation and recruitment of monocytes/macrophages to mucosal sites as the result of a local infection. These results raise the importance of the development of an appropriate innate immune response at the site of infection in order to obtain an effective immune response against this parasite.

BENEFITS AND ADOPTION

This project benefits the Tasmanian Atlantic salmon industry by contributing to improved knowledge of *Neoparamoeba* spp and to a better understanding of host-pathogen interactions. This knowledge can be applied in future work in vaccine development and on prevention of AGD. For example, the finding that components of the glycocalyx of the parasite exert a "suppressive" effect on the immune response of salmon to *Neoparamoeba* spp highlights the likelihood that the parasite is using these molecules to avoid a protective immune response, thus making salmon more susceptible to AGD infection. Any vaccine formulation will have to take into account this effect and include adjuvants that can overcome this negative effect. In addition, this finding opens another field of research on possible treatments that target the "immunosuppressive" component(s) produced by the parasite.
This project also contributed to the general area of AGD research by producing a panel of monoclonal antibodies that can be used as tools for the identification of different strains and/or species of *Neoparamoeba* in the field, and for further elucidation of the complex cell surface composition of the parasite.

FURTHER DEVELOPMENT

Further investigation of the immunomodulatory effect of the HMWA from infective *Neoparamoeba* should be a high priority as this may play a critical role in the pathogenicity of the parasite. For example it would be very useful to compare the transcriptome of fish immunised with different fractions of HMWA with the trancriptome of "normal" gill tissue and gill tissue from infected fish. Such a study would complement the work already undertaken by colleagues at TAFI (Young et al., 2008) who have shown down regulation of immune related molecules in salmon infected with *Neoparamoeba*. This work could then be extended to assess the effect of isolated components of HMWA on salmon macrophages *in vitro.* If the immunomodulatory effect of this fraction is confirmed *in vivo* or *in vitro* then detailed knowledge of the immunomodulatory factor(s) may provide a novel target for vaccine development or treatment.

The high molecular weight fraction tested in the immunisation trial carried out in this project may also contain protective antigens, such as attachment factors, the potential of which has been overshadowed by the immunomodulatory component also contained in this preparation. Separation of the different components of this fraction would facilitate such an analysis.

The finding that treatment with adjuvant alone can provide, in certain circumstances, a significant level of protection against AGD warrants further investigation to determine which immunological parameters are important in this phenomenon. The possible effect of co-infection with a mucosal pathogen could also have an impact on future vaccine development strategies

PLANNED OUTCOMES

General objective: to develop a set of strategies and tools to enable the salmon industry to achieve a substantial reduction in the economic impact of amoebic gill disease; Clear evidence of feasibility of native or subunit vaccine.

The planned outcomes of this project focused on the identification of proteins involved in AGD pathogenesis including both attachment molecules and/or antigens that are differentially expressed in infective and cultured amoebae to produce a subunit vaccine to protect Atlantic salmon from AGD.

Using MAbs that react specifically with infective *Neoparamoeba* we have identified a high molecular weight fraction from the parasite that contains a component or components that are involved in the attachment of the parasite to host tissue. To identify these molecules we produced MAbs specific to the surface of the infective parasite. As a result of this work we demonstrated that the main difference between the surfaces molecules of infective and non-infective parasites were carbohydrate epitopes expressed on high molecular weight glycoproteins. The production of an effective carbohydrate based vaccine has traditionally been considered very difficult but new advances in glycobiology indicate that a conjugated vaccine targeting carbohydrate antigens is now a real possibility. Therefore, we tested this high molecular weight fraction as a potential vaccine. In the course of this study we identified the presence of an immunomodulatory component that down regulated the immune response in salmon. This immunomodulatory activity is highly significant with respect to the future development of an optimised vaccine against AGD. We also found that the stimulation of the innate immune system by administration of adjuvant or co-infection may provide a significant level of protection against AGD.
On the broader front, this project has provided a set of tools in the form of a panel of monoclonal antibodies that can be used for further characterisation of the antigens expressed by *Neoparamoeba.*

CONCLUSIONS

Objective 1 - To identify potential protective antigens from *Neoparamoeba* spp using a combined DNA/protein approach.

We have produced and screened an extensive panel of MAbs using strategies to obtain MAbs that recognise surface proteins unique to infective parasites. But the high percentage (97%) of MAbs specific for carbohydrate epitopes obtained, even when deglycosylated membrane was used as the immunogen, suggested that the majority of the surface molecules unique to WT parasites are carbohydrate in nature. The only two MAbs obtained that recognise peptide epitopes on surface proteins were cloned and sent to CSIRO to be used as tools for the screening of cDNA libraries generated by that group. In addition, one MAb, an IgM specific for a peptide epitope expressed in the cytosol and unique to WT parasites was also sent to CSIRO.

Our subsequent work focussed on the identification of molecules of any nature with potential for eliciting a protective immune response in salmon. A high molecular weight antigen (HMWA) derived from WT parasites was identified as containing components involved in the attachment of parasites to the gill epithelium and hence as having potential as a target for a vaccine against AGD. Antigens in this fraction were recognised by a large number of the WT-specific MAbs reacting with carbohydrate epitopes on the surface of the parasite. These MAbs showed positive inhibition results in the *in vitro* attachment assay. An immunisation/challenge trial was performed to test the vaccine potential of this fraction.

Objective 2 - To identify and characterize attachment molecules involved in the infection process of Atlantic salmon by *Neoparamoeba* spp.

Two polyclonal rabbit sera, raised against whole WT parasites and a membrane fraction of WT parasites respectively, and a total of 58 MAbs, recognising antigens expressed on the surface of the parasite, have been evaluated and relevant antibodies were tested for their capability to block the attachment of the parasite to salmon gill epithelium in an *in vitro* attachment assay. Both polyclonal sera gave a negative result in the *in vitro* attachment assay. Three MAbs demonstrated statistically significant capacity to inhibit the attachment of parasites to gill explants. The levels of inhibition ranged from 51 to 77% compared to the isotype-matched negative control. The three MAbs recognise carbohydrate epitopes on a cluster of antigens present in the size exclusion high molecular weight fraction (HMWA) of infective parasites that represent 19% of the total protein in the soluble fraction of the parasite. Treatment of this HMWA with TFMS indicated that the carbohydrate portion constitutes more than 70% of the total molecular weight of these molecules. While a similar HMWA complex is present in non-infective parasites, these glycoproteins are not recognised by any of these three MAbs.
The 4 glycoproteins recognised by these MAbs are resistant to standard enzymatic deglycosylation. These four glycoproteins are bound by disulfide bridges to form complexes of *M*r greater than 600 kDa. Attempts to further characterise the multiple components in the HMWA have focussed on analysis of the protein cores. Deglycosylated material has been fractionated by HPLC to yield four protein components that have each been subjected to analysis by mass spectrometry.

Objective 3 - To demonstrate protection of Atlantic salmon against clinical AGD via cDNA and/or recombinant protein vaccination

Two immunization/challenge experiments were performed in this project. In the first the HMWA described above was used to immunise salmon and the potential of this preparation to protect from challenge with infectious *Neoparamoeba* spp was assessed. In this experiment all fish immunised with HMWA responded with significant antigen-specific serum antibody titres at day 49 while significant antibody levels were not detected in fish from the control groups (inoculated with PBS emulsified in Freund's Complete Adjuvant or PBS alone). In contrast to the response detected in serum, specific antibodies were not detected in the mucus of any fish at day 49. However, a low but detectable level of antibody was present in the mucus of the five HMWA immunised fish that survived until the termination of the experiment.

Analysis of the survival of fish in the 3 groups following challenge with infective parasites showed that despite the presence of specific serum antibody, fish immunised with the whole HMWA fraction were not protected from infection with the parasite. Indeed, fish immunised with adjuvant alone exhibited a survival advantage over non-treated control fish and fish immunised with HMWA plus adjuvant. This latter finding suggests that the HMWA may contain components that suppress a protective immune response. The HMWA fraction is a mixture of macromolecules, some of which may be potentially protective while others may possess "immunosuppressive" activity. If this is the case the "immunosuppressive" component may be masking any potential protective effect that may be achieved by targeting the molecules recognised by MAb 44C12.

The surprising observation that fish in the adjuvant control group exhibited significantly lower mortality compared to both the antigen immunised group and fish injected only with PBS lead to a second immunization/challenge experiment designed to assess the potential of Freund's Complete Adjuvant and Freund's Incomplete Adjuvant alone to protect salmon from challenge with infectious *Neoparamoeba*. The results of this second trial showed no differences between the experimental groups. The different results obtained in the two trials may be explained by the fact that in the first trial some fish in the tank were infected with a skin pathogen. This co-infection probably helped in the recruitment of activated monocytes/macrophages (systemically activated by the adjuvant) to the mucosal sites; a recruitment that otherwise would be very poor since the parasite itself doesn't induce a significant inflammatory response at the gills.

The fact that fish immunised i.p./i.p. with HMWA developed strong speciific systemic antibody responses but not a local immune response at the gills. The suggestion that a mucosal stimulus was necessary for the protective effect of FCA reinforces the conclusion that different immunization strategies need to be developed to elicit an appropriate immune response at the local level.

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APPENDIX 1: INTELLECTUAL PROPERTY

A panel of monoclonal antibodies exhibiting differential reactivity patterns for infective and non-infective *Neoparamoeba* spp.

Selected monoclonal antibodies that may be used to identify potential targets for development of an AGD vaccine.

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APPENDIX 3:

CSIRO Statistical Analysis Report

UTS AGD Vaccine Tank Trial – December 2006 / January 2007

In December 2006 - January 2007 a tank trial was run to examine the effectiveness of vaccine against AGD. There were 73 fish placed in a single tank, with AGD infection introduced on 15 December.

The fish were assigned to four 'treatment' groups:

Treatment	**Description**	**No. fish**
HMWA + FCA	Treatment group which received the vaccine	10
FCA + PBS	Received adjuvant only	11
PBS only	Handling control	14
AGD infection control	Unhandled fish, to make up the required density	38

Observations of fish mortality were made until 23 January, when the experiment was terminated.

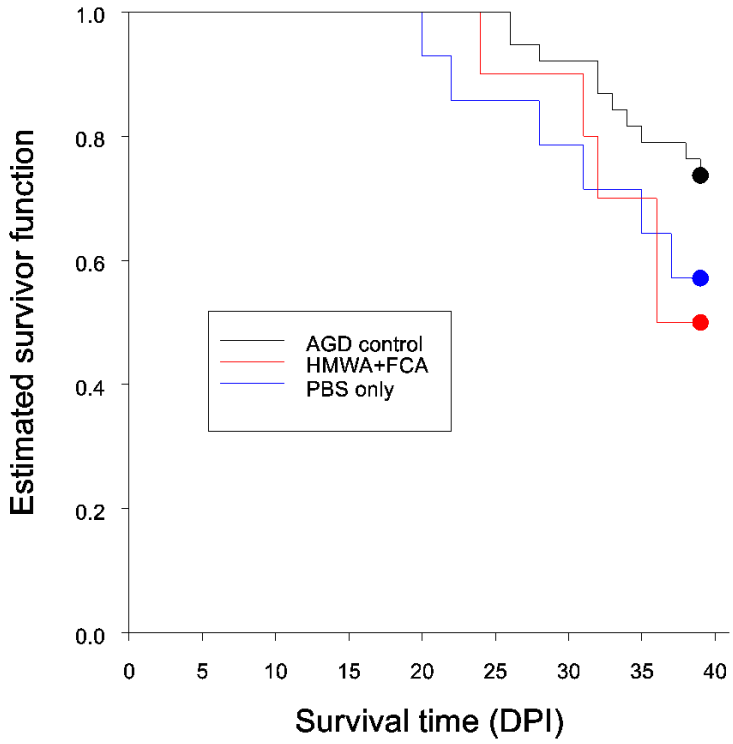
The results of the experiment were:

		Deaths at Each Date			
Date	**DPI**	**HMWA + FCA**	**FCA + PBS**	**PBS only**	**AGD infection control**
3/01/2007	19	0	0	0	0
4/01/2007	20	0	0	1	0
6/01/2007	22	0	0	1	0
8/01/2007	24	1	0	0	0
10/01/2007	26	0	0	0	2
12/01/2007	28	0	0	1	1
15/01/2007	31	1	0	1	0
16/01/2007	32	1	0	0	2
17/01/2007	33	0	0	0	1
18/01/2007	34	0	0	0	1
19/01/2007	35	0	0	1	1
20/01/2007	36	2	0	0	0
21/01/2007	37	0	0	1	0
22/01/2007	38	0	0	0	1
23/01/2007	39	0	0	0	1
Survivors		5	11	8	28
Total		**10**	**11**	**14**	**38**

Kaplan-Meier Survivor Function

The survivor function is relationship between the probability of an individual still surviving and the elapsed time since infection was introduced. The Kaplan-Meier survival function was fitted to the three groups where some mortality occurred, using the method described by Kalbfleisch and Prentice (1980). These functions are presented in Figure 1.

Figure 1. Kaplan-Meier estimates of the survivor functions



Once deaths started occurring from DPI 20, the survival functions are approximately linear. On this graph, the survivor function for the treatment with no mortality, FCA + PBS, would be a straight line at y = 1.0.

Cox Proportional Hazards Model

The proportional hazards model (Cox 1972) is the standard technique used to fit survival data and test between treatments. This model was initially fitted to the full set of data, i.e. all four treatment groups. There was an overall significant difference between the groups (deviance = 10.79; d.f. = 3; P = 0.013).

The AGD infection control fish, whose hazard function was between those for FCA+PBS and for the other two treatments, were treated differently in that they weren't handled and were really only present to make up the required density of fish. Hence the proportional hazards model was fitted omitting the AGD infection control fish. It still showed an overall significant difference between the three groups where handling occurred (deviance = 10.18; d.f. = 2; P = 0.006).

Consequently it is reasonable to conclude that the mortality of the FCA+PBS treatment is significantly different to that of the other two treatments.

Exact Test on Final Mortalities

Noting the near-linearity of the survivor functions in Figure 1, the outcome of the experiment can reasonably be summarised by the final mortalities:

Treatment	Dead	Alive	Total No. fish	% mortality
HMWA + FCA	5	5	10	50
FCA + PBS	0	11	11	0
PBS only	6	8	14	43
AGD infection control	10	28	38	26

Again ignoring the AGD infection control fish, which were not handled, an exact test was performed on the 2 x 3 table of dead and alive numbers of fish. This test calculates the probability that the observed counts could have arisen by chance alone, using a simulation technique of Patefield (1981).

The test was run on 20000 simulations and gave a significant result; P = 0.0135.

Conclusion

The different tests performed have all shown a significant difference between the treatment groups. The AGD infection control fish have mortality (26%) between that of the group that received adjuvant only (0%) and those which were handled only (43%) and vaccinated (50%).

This experiment provides no support for the vaccine being effective against mortality, and poses the question why fish with adjuvant only had significantly lower mortality than other handled fish.

The experiment was performed in a single tank with very small numbers of treated fish for testing a binary response, i.e. dead or alive. To establish the result with greater confidence, much larger numbers of fish would be needed and preferably more than one tank used.

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Warren Müller
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APPENDIX 4: SCIENTIFIC COMMUNICATIONS

Publications

Adams M., Villavedra M. and Nowak B., 2008. An opportunistic detection of amoebic gill disease in blue warehou , *Seriolella brama* Gunther, collected from an Atlantic salmon *Salmo salar* L., production cage in south eastern Tasmania. Accepted to be published in *Journal of Fish Diseases.*

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Manuscripts in preparation

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