VACCINATION OF ATLANTIC COD (*Gadus morhua* L.) AGAINST ATYPICAL FURUNCULOSIS USING DIFFERENT ADJUVANTS

by

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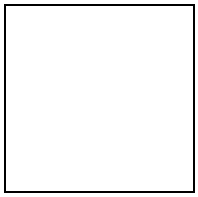

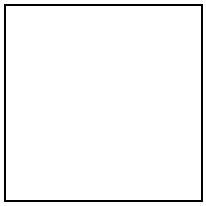

ꕥ *Dedication* ꕥ

I dedicate this work to my father, mother, sisters, and brothers.

4th May 2003

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"Praise be to Allah"

This work was carried out in the period from October 2002 to May 2003 at the Department of Marine Biotechnology at the Norwegian College of Fishery Science (NCFS) and was apart of a project financed by the Norwegian Industrial and Regional Development Fund (SND). The project is a co-operative project between NCFS and Fiskeriforsking.

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ABSTRACT

In recent years increased attention has been focussed on fish vaccines and vaccination against diseases in farmed fish. In this study, efficacy and side effects of vaccination against atypical furunculosis in Atlantic cod (*Gadus morhua* L.) were studied in an experimental trial by using different adjuvants. The different adjuvanted vaccines were administered by intraperitoneal injection (i.p.) to Atlantic cod. The adjuvants used were CpG DNA, Aluminium hydroxide (Alhydrogel), and Freunds incomplete adjuvant (FIA). Efficacy of the vaccines and side effects were determined 12 weeks post vaccination, whereas serum antibodies were measured 15 weeks post vaccination.

Vaccination of Atlantic cod against atypical furunculosis protects the fish and elicits specific antibody responses to experimental infection with atypical *A. salmonicida*. The atypical *A. salmonicida* bacterin alone resulted in very good protection (RPS=75%), and addition of adjuvant alone or in combination did not improve the protection significantly. This indicates that Atlantic cod can be protected against atypical furunculosis through non-adjuvanted vaccines. The injection of FIA alone gave non-specific protection, while the adjuvants CpG DNA and Aluminium hydroxide did not give any non-specific protection. The group, which received CPG DNA alone, had a higher mortality (57%) than the saline control group (53%). High specific antibody responses were demonstrated in all groups vaccinated with *A. salmonicida* bacterin. The responses were strongest in the groups, which received A. *salmonicida* in FIA.

The study also assessed the side-effects 12 weeks vaccination. The vaccines containing FIA showed the most sever side-effects among the vaccinated group. Aluminium hydroxide showed a less degree of the intraabdominal side effects compared to FIA, but a delay in fish growth was observed.

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INTRODUCTION

1.1 General introduction

During the past three decades, aquaculture has developed to become the fastest growing food producing sector in many parts of the world and has become the characteristic feature of today's fishery industry. In aquaculture, according to the intensive system of production, fish are kept in high densities and the possibility for exposure to disease pathogens[1], which can be bacterial, parasitic, or viral, throughout production cycle is becoming very high (Munro *et al*., 1993). Under such conditions, the problems of the infectious fish diseases become serious and have considerable effects. In fact, bacterial and viral diseases of farmed fish have lead to high moralities and reduced economical income for the fish farming industry (Pilcher *et al*., 1980). Furthermore, Inglis *et al*., (1993) stated that fish diseases, particularly those caused by bacterial pathogens are the most important causes of losses among fish farm stocks. Many pathogens have caused severe, even catastrophic, losses in aquaculture industry. For example, infectious salmon anaemia (ISA) virus nearly put the Norwegian salmon aquaculture in a real crisis in recent years. Also, bacterial infectious diseases, like vibriosis (*Vibrio anguillarum*) and furunculosis (*Aeromonas salmonicida*), have caused serious problems for the salmon industry in Scandinavia (Egidius, 1987). There are many more examples, but what has been mentioned reflects the problem.

In line with the increased economic importance of aquaculture world wide, vaccines against several bacterial fish diseases have been developed during the last 15 years, vaccination has become an important method to prevent infectious diseases in aquaculture industry. Vaccines against many infectious fish diseases, for example vibriosis, furunculosis, and red mouth disease (ERM), have become commercially available and are very effective in giving protection.

Furunculosis and atypical furunculosis are both serious and economically important infectious bacterial diseases in salmonids and non-salmonids farming industries (Thronton, 1995; Magnadottir *et al*., 2002). In the Norwegian salmon farming industry, furunculosis has caused large economic losses at the end of 1980's and as a consequence all farmed salmon are today vaccinated against furunculosis. The

[1] Micro-organisms which infect the fish body and cause diseases (causative agents of the diseases).

available vaccines against furunculosis are administered by intraperitoneal injection (i.p.) as a multi-component vaccine containing *A. salmonicida* antigen and mineral oil. A remarkably long-lasting protection is shown by the vaccines (Ellis, 1997) and as a result of intensive vaccination, the incidence of the disease among salmonids has been dramatically reduced.

Atlantic cod (*Gadus morhua* L.) is an economically and historically (Kurlanski, 1998) important species in the northern Atlantic and in the Norwegian fishery. During the last couple of years, there has been a trend of introducing new non-salmonid fish species for farming purposes of intensive culture in several countries, for example in Iceland, Canada, Norway and Scotland (Tilseth, 1990). The Norwegian aquaculture industry, which is based on salmonids will consist of much more than salmon and trout farming in the future, such as Atlantic cod, halibut (*Hippoglossus hippoglossus*), spotted wolfish (*Anarhichas minor* O.), and other marine fish species (www.fiskeopprett.no., (2001)). Furthermore, the interest in the intensive culture of Atlantic cod has increased dramatically due to a reduced supply from the wild fishery, which leads to a high demand for this species on the market. However, as all other farmed fish species, cultured cod has showed cannibalism in the early stages of culture (Folkvord, 1992; Rosenlund *et al*., 1993) and susceptibility to infectious diseases (Espelid et al., 1991; Schrøder *et al*., 1992; Wiklund and Dalsgaard, 1998). Therefore, in the context of an expanding industry, many issues are raised in cod health and disease control measures to enhance protection of cod against the infectious diseases.

Atypical furunculosis is caused by atypical *Aeromonas salmonicida*, and has been detected in salmonids and non-salmonids in a wide range of both freshwater and marine fish species (Conick *et al*., 1984; Austin and Austin, 1993; Gudmundsdottir 1998; Wiklund and Dalsgaard 1998). In Iceland, atypical furunculosis is the main disease in farmed salmonids and has caused serious losses before vaccination became a common practice (Ellis, 1997). Several outbreaks of atypical furunculosis disease in Atlantic cod have also been reported and in other species (reviwed by Wiklund and Dalsgaard 1998). Therefore, vaccines designed for cod is under investigation. The cod farming industry will possibly face the same disease problems as the salmon farming industry experienced twenty years ago and because of this, it is of crucial importance to gain as much information about effective vaccines against cod diseases as possible.

There are different types of adjuvants that have been used in commercially produced fish vaccines (Anderson, 1997). One of the most commonly used adjuvants is mineral oil, which creates a slow-release depot of the antigen in the body lumen, resulting in a prolonged time for which the immune system remains in contact with the antigen. In addition, adjuvants stimulate the non-specific part of the immune system (Ellis, 1988). However, in many cases these adjuvanted vaccines tend to elicit different intra abdominal lesions at the site of injection (Munn &Trust, 1983; Cossarini, 1985) and because of these side-effects researchers are investigating adjuvants that augment protective immune responses without or with minimal side-effects, by comparing various adjuvants (Midtlyng *et al*., 1996a; Klinman *et al*., 1999; Weerantna *et al*., 2000).

The work described in this paper forms part of a project, which aims to investigate protection against atypical *A. salmonicida* in Atlantic cod. One of the goals in this project is to examine the use of other adjuvants than oil in cod vaccines and their protective immunity against atypical furunculosis.

1.2 Atlantic cod biology

1.2.1 Species

Cod is the common name for nearly 60 species of valuable food fish of the family Gadidae (King, 1995), order Gadiformes. Other families in the order are also known as cod, such as the deep-sea cod of the family Moridae, but the best-known and most commercially important cod is the Atlantic cod, *Gadus morhua*, of the family Gadidae (King, 1995).

The Atlantic cod has three dorsal fins, two anal fins, an unfrocked tail, and a small barbel on its lower jaw (Fig.1). It is generally moderate in size, but can weight as much as 90 kg, and can be as long as 150 cm (King, 1995; Macer, 1991). The colour of the cod is Greenish-grey to blackish-brown and sometimes red, and it has a marbled pattern on its head, back, and sides.

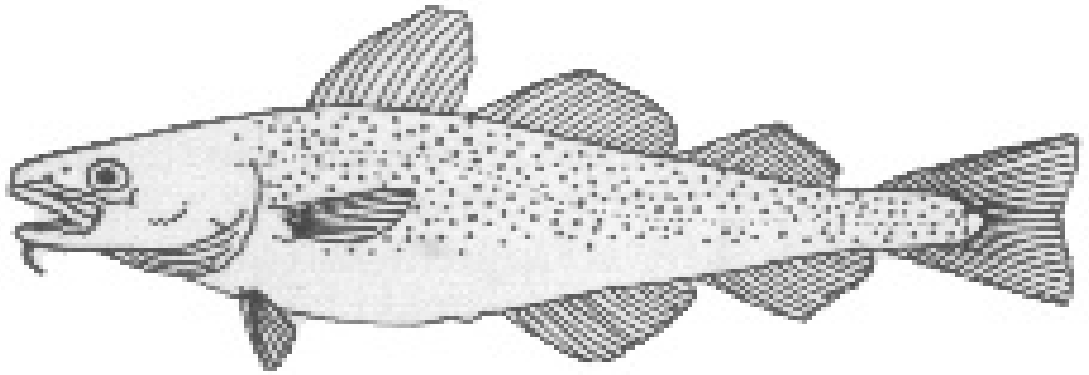


Figure 1.1 Atlantic cod (*Gadus morhua* L.)

1.2.2 Distribution and Habitat

The Atlantic Cod are a cold-temperate gadoid, distributed in cool waters in the Northern Hemisphere. Although they are bottom dwellers, cod can be found anywhere from the surface to 500 or 600m and from inshore waters to the edge of the continental shelf. The range of the Atlantic cod spans most of the coastal waters of the north Atlantic. Atlantic cod are found in the Northeast Atlantic, from Iceland to the Norwegian Sea and south to the Baltic Sea and the Bay of Biscay (Jackobsen, 1998; Bergstad *et al*., 1987). In the North West Atlantic their range extends from Greenland and south Baffin Island, south along the continental shelf to the Gulf of Maine. (http://www.science.mcmaster.ca/biology/4S03/ATCOD-1.HTM). The normal temperature range for cod is between –0,5 to 10^{o}C, and it depends on the time of the year, location, and the size of the fish. They can be found in waters up to about 20^{o}C.

1.2.3 Reproduction

Cod show long distance migrations between spawning, feeding and over wintering habitats (http://www.umaine.edu/aquaculture/cod/ecology/spawning.htm).

Cod are total spawners, which means all eggs are produced and spawned at one time during the year. In general, fish of the cod family are planktonic spawners, producing buoyant, pelagic eggs that mix with the plankton and float freely (Kjesbu, 1989). The spawning of cod in the northwest Atlantic is therefore timed with plankton production (http://www.umaine.edu/aquaculture/Cod/Ecology/Spawning.htm).

The cod gather in large numbers during the winter months to spawn. They migrate to a number of spawning grounds and release their eggs in depth of about 200m (King, 1995). Every egg has a droplet of oil so that it floats and wildly spread by currents.

The larval cod become part of the plankton for about 10 weeks. They sink to the bottom when they are about 2 cm long, begin to migrate in the second year, and spawn when five to eight years old (Kjesbu, 1989).

1.2.4 Feeding

Atlantic cod is a voracious predator, feeding on the bottom living fish and invertebrates (Macer, 1991). The size of the fish plays an important role in what they will eat since they have an enormous food range. Food preference changes as the cod grows. Young cod fry feed mainly on copepods, amphipods, and other small planktonic crustaceans. Juveniles feed mainly on shrimp, amphipods, euphaussids, and on fish and shellfish larvae. Adult cod feed mainly on Capelin, herring, sand lauce, founders, young tourbout, crabs, shrimp, brittle stars, combjellies, small cods and a host of other species of fish and shellfish.

1.2.5 Status of the stock

Atlantic cod has been the most important fish species for centuries and has played an important role in the development of countries around the North Atlantic (Kurlanski, 1998). Furthermore, cod is valued as the source of cod-liver oil. They form the basis of a historically profitable fishery in the North Atlantic. Such fishing is still an important part of the economy in many countries such as Norway, Iceland, Great Britain and Canada. Bottom trawling, Danish seine nets, longlines, handlines, and pots are all fishing gears used to harvest cod. Thinning of the fish stocks, however, has made the culturing of Atlantic cod more viable (Tilseth, 1993). In the early 1980s, the production of Atlantic cod fisheries decreased as the cod stocks in the Northern Atlantic were severely depleted. Today, despite the efforts to rebuild and protect the cod population through application of different management measures, Atlantic cod stocks are still being over harvested.

1.2.6 The immune system of the Atlantic cod

Atlantic cod possess the same major immunological organs as other teleost species, which comprise of head kidney, thymus and spleen (Press and Evensen, 1999: Schrøder, 1998). In general, the fish immune system consists of a specific and non-specific part. The features of the specific immune system are antigen specificity and immunological memory. This means that most or all antigens can be recognised by

the specific immune system. Further more, this enables all fish species, including cod, to be vaccinated against particular antigens.

According previous publication, injection of bacterial antigens leads to an elevated level of specific antibody in fish (Bøgwald *et al.,* 1991; Erdal and Reitan, 1992; Estvez *et al*., 1994; Eggset *et al*., 1997). However, these specific responses are not observed in cod against *V. salmonicida* and protein antigens (Pilström and Petersson, 1991; Schrøder, *et al*., 1992; Magnadóttir et al., 2001) whereas only limited responses was observed against *V. anguillarum* (Espelid *et al*., 1991). The serum concentration of cod IgM is found to be high compared to salmon and haddock (Israelsson *et al*., 1991) and as in other fish species the immunoglobulins in cod are characterised as a tetrameric IgM molecule (Pilström and Petersson, 1991). Thus, cod is capable to produce immunoglobulins like other fish species.

Several studies of the genetic mechanisms, which create antibody diversity, have not shown other mechanisms in cod compared to other fish species (Pilström and Bengtén, 1996; Pilström and Petersson, 1991; Stenvik, 2001). No special features were found in the H-chain variable region (VH) repertoire characterised by cDNA cloning and sequence analysis (Stenvik *et al*., 2000) and cloning and analysis the cod IgD showed similarity to the delta domains in Atlantic salmon (Stenvik and Jørgensen, 2000).

Because of the difficulties in producing an elicited antibody response in cod it is suggested that may be cod depends on other mechanisms to encounter the pathogens than other fish species (Israelsson *et al*., 1991; Daggfeldt, 1994; Schrøder *et al*., 1998).

1.3 Atlantic cod aquaculture

Aquaculture of Atlantic cod is a new industry in several countries, including Norway, Britain, Canada and Iceland, (Walden, 2000). The most significant challenge of intensive rearing of cod has been a continuous production of large amounts of cod larvae under controlled conditions. In recent years, cod producers have made promising progress in this field and in 2002 more than three million cod have been produced in Norway (www.intrafish.com/intrafish-analysis). It is obvious that the significant advancement in the intensive culture of this species depends on its larval production. Furthermore, the commercial value of cod encourages farmers to culture this species. Therefore, the future looks good for intensive Atlantic cod aquaculture

and cod farming will probably become a viable industry in the near future (Steinarsson and Bjørnsson, 1999; Bjørnasson *et al*., 2001). However, Atlantic cod, like other fish species, in the intensive culture system, are susceptible to a number of pathogens.

Today, classical viriosis is the main disease problem in cod aquaculture, but outbreaks of both atypical furunculosis and coldwater vibriosis have occurred (Willumsen, 1990; Schrøder *et al*., 1992; Wiklund and Dalsgaard, 1998).

1.4 Atypical furunculosis and classical furunculosis

Furunculosis is a serious infectious bacterial disease for both salmonids and non-salmonid fish species and is characterised by mass mortality among the infected fish population. In 1989, furunculosis was endemic in several parts of Norway and caused severe losses in the salmonids farming industry.

The causative agent of the disease is the gram-negative bacterium, *A. salmonicida* (Munro and Hastings, 1993), which is a well-known pathogen in fish. *A. salmonicida* has been classified into subspecies namely, *salmonicida, achromogenes,* and *masoucida, smithia,* and *pectinolytica* (Popoff 1984; Pavan *et al*.,2000).

Typical furunculosis is caused by homogeneous strains of *A.salmonicida* subspecies *salmonicida*. Atypical furunculosis is caused by heterogeneous strains of *A. salmonicida* subspecies *achromogenes, masoucida, smithia,* and *pectinolytica* (Smith, 1963; Austin *et al*., 1989; Pavan et al., 2000). Recently, atypical *A. salmonicida* has been isolated from wild and cultured cod (Wiklund and Dalsgaard, 1998; Magnadottir *et al*., 2002; Lund *et al*., 2002b). In addition, atypical furuculosis seems to be more conspicuous in farmed fish than in wild fish with some host specific virulent variation (Wiklund and Dalsgaard, 1998; Lund *et al*., 2002b). Symptoms of atypical furunculosis disease in cod are mainly characterised by skin ulceration and the formation of furuncles in the later stages (Magnadóttir *et al*., 2002). The disease can be either acute or chronic and high mortality rates are associated with both infectious forms as in salmonids (Bruno *et al*., 1996). Other symptoms include raised bumps underneath the scales and these furuncles contain large numbers of A. *salmonicida* bacteria, which can spread in the water and has been considered to be a source of infection (Enger *et a*l., 1992). In the chronic stage of the disease, the furuncles create large bleeding ulcers (Munro *et al*., 1993).

Form of transmission and survival of atypical *A. salmonicida* in water is not yet known and the source of infections remains uncertain (Gudmundsdottir, 1998). The bacteria are found naturally in marine waters (large numbers) and it seems very possible that cod could be infected directly from the marine environment. Diseased fish may act as a source of infection (Magnadóttir *et al*., 2002).

1.5 Fish vaccination

1.5.1 Introduction

" Vaccines are preparation of antigens derived from pathogenic organisms, rendered non-pathogenic by various means, which will stimulate the immune system in such a way as to increase the resistance to disease from subsequent infection by a pathogen"

Ellis (1988)

The purpose of vaccination is to induce protective immunity in an animal. The immunity is specific and should be long lasting. Vaccination is prophylactic and is much more effective against infectious diseases compared to other types of treatments, for example antibiotic treatment. In aquaculture, the vaccine should be effective and applicable against the most economically important diseases. The commercially available vaccines used against fish bacterial diseases contain inactivated bacterial cells. In addition, the vaccines contain adjuvants, which increase the efficacy of the vaccines. The commercially available salmon vaccines in Norwegian aquaculture are oil-based adjuvanted vaccines and are most often multivalent vaccines, containing up to seven different components.

The current advances in technology seem to offer a promising future for anti-bacterial vaccines in fish species. However, it is more difficult to develop effective and cheap anti-viral vaccines and anti-parasitic vaccines.

1.5.2 Route of administration

Vaccines can be administered by injection, immersion, or by oral administration. Simply, vaccines can be administered by adding the vaccines to food. The disadvantage of this method is the usage of large amounts of antigen within an accurate dose of food for each fish (Gravningen *et al*., 1993; Bøgwald *et al*., 1993). In addition, the acidic environment of the digestive tract may deactivate the vaccine. In spite of the above-mentioned limitations, orally administered vaccine has achieved in

some countries, effective oral vaccine against enteric red mouth disease (ERD) is used for fish, (fish farmer, 2002).

The immersion administration is useful for mass vaccination especially for small fish. Farmers preferred this method because of it is cost effective (Horne and Ellis, 1988). The vaccine is applied externally to fish using different techniques including spray, direct immersion and flush exposure (Anderson *et al*., 1983). The antigen then enters the fish body through the skin or the gills. The concentration and the duration of exposure are important factors, which affect the conclusive results regarding the protection using this method. In addition, in spite of more than 15 years of effort with anti-furunculosis immersion vaccination, the result did not give proper protection (Midtlyng, 1998a).

The injection administration gives the best and most long-lasting protection. The vaccine can be applied by injecting the fish either intra peritoneal (i.p) or intramuscularly (i.m). Also, injection has the advantage of delivering the right dose of vaccine antigen, (Ellis, 1998; Smith, 1988). Injection is the most used method however, it is quite expensive due to the intensive labour forces needed to handle fish during the vaccination process.

1.5.3 Types of vaccines

Vaccines can be developed in different ways so that it can develop protective immunity against the pathogen in the vaccinated fish. The vaccine components can be prepared as inactivated whole cells, DNA vaccines or recombinant or subunit vaccines.

The whole cell inactivated vaccines have a significant advantage over other types in their safety and easy development. Almost all the commercially available vaccines are inactivated whole bacterial cells. These vaccines are effective and protect fish against diseases (Ellis, 1997; Stevenson, 1997). During the recent years also inactivated virus vaccines are developed against infectious pancreatic necrosis (IPN) in Atlantic salmon (Dixon, 1997).

The subunit vaccines consist of parts of a pathogen that stimulate the protective immunity. Vaccination of Atlantic salmon with recombinant infectious pancreatic necrosis virus (IPNV) subunit vaccine induces production of IPNV specific antibodies protection. The subunit technique involves the isolation of the glycoprotein gene, which encodes the viral protein responsible for inducing a protective immune

response in fish (Winton, 1997). DNA vaccines, produced by recombinant DNA technology, represent a new area of vaccine development and have been shown to be effective to produce immunity to several viral diseases, as infectious pancreas necrosis (IPN) and viral haemorrhagic septicaemia virus (VHSV) (Frost and Ness, 1997) According to Gudding *et al*., (1999) DNA vaccinations have advantages over conventional vaccines because the specific immune response after using DNA vaccination include the antibodies, T-helper cells and cytotoxic cells. However, before vaccines are applied commercially in aquaculture, safety for fish and the environment have to be addressed.

1.5.4 Vaccination against fish diseases

Before the introduction of vaccines in aquaculture, bacterial diseases of fish were controlled in many countries by the use of a wide range of chemotherapeutics i.e. antibiotic therapy (Hastings, 1997). In Norway, 47.000 kg of antibiotics in 1987 for disease control in salmon farming industry (Markestad *et al*., 1997). This resulted in development of antibiotic resistance in many fish pathogenic bacterial strains. Furthermore, there were increasing concerns on the level of antibiotics in fish flesh intended for human consumption. According to Inglis *et al*., (1993) residues of antibacterial treatment in fish have had an indirect impact on human health. This also, has had environmental impact on aquatic species.

The increasing problems associated with the usage of antibiotics to control fish diseases encourages researchers to investigate the possibility of developing vaccines against the major diseases of farmed fish and to emphasise the idea that prevention of disease is more important than therapy.

The first commercial fish vaccines were licensed in Europe and Scandinavia in mid 1980's and were protecting against enteric red mouth disease (ERM) and vibriosis (Ellis, 1988). The use of these vaccines has greatly reduced the level of infections in salomonids and other farmed species. Furthermore, furunculosis vaccines were licensed in Europe in 1986; and were introduced to the Norwegian market in 1989 (Markestad *et al*., 1997).

1.5.5 Adjuvants[2]

According to Ellis (1988) an adjuvant can be defined as a "substance administered with antigen which non-specifically enhances the immune response". According to Anderson *et al*., (1997) adjuvants are additives to an antigen, which enhances the immune response in fish, resulting in an effective prolonged protection. Therefore, addition of adjuvants to fish vaccines for increasing its effectiveness against furunculosis or other fish bacterial diseases is important and has been studied for a long time in fish vaccine research (Paterson, 1985; Hastings, 1988; Anderson, 1997). There are different kinds of adjuvants, such as mineral oil, freunds complete/ incomplete adjuvant (FCA /FIA), glucans, and aluminium salts, which have been used in fish vaccines (Anderson *et al*., 1992 &1997; Robertson *et al*., 1994). The commonly used adjuvants is mineral oil, which create a slow-release depot of the antigen in the body lumen, resulting in a prolonged time for which the immune system remains in contact with the antigen (Ellis, 1988). The oil adjuvanted injectable vaccines against *A. salmonicida* (multivalent or monovalent) give long-lasting protection in salmonids and other marine fish species (Midtlyng *et al*., 1996 a, b; Ingilæ, *et al*., 2000). However, in many cases these adjuvants tend to elicit different intra abdominal lesions at the site of injection (Munn and Trust, 1983; Cossarini, 1985). In addition, depress in growth and qualities of farmed salmonid species are observed (Midtlyng and Lillehaug, 1998b).

FIA is a mineral oil adjuvants used in fish vaccines. Intraperitoneal injection of FIA based vaccines give significant enhancement of the immune response and high level of protection in vaccinated fish. However, it causes serious visible intra abdominal lesions, including visceral adhesion, non-transparent membranes and reduction of the growth rate (Midtlyng *et al*., 1996a; Midtlyng and Lillehauge, 1998b).

The use of CpG DNA as an adjuvant is a new area in general and in particular in fish. In a recently published papers, it has been shown that CpG adjuvant used in fish vaccination is a very promising tool for prophylaxis against many bacterial infectious diseases in aquacultured fish and other animals model (Weiner *et al*., 1997; Jørgensen *et al*., 2001; Krieg, 2000; Krieg, 2002).

Bacterial DNA contains unmethylated CpG motifs, which are more often present in bacterial DNA than vertebrate DNA. According to Jørgensen *et al*., (2001),

[2] Latin word mean " to help".

unmethylated CpG ODNS leads to production of antiviral cytokine activity in Atlantic salmon (*Salmon salar* L.) leucocytes. In addition, studies of rainbow trout (*Oncorhynchus mykiss*) have reported the production of interferon like cytokines in the head kidney (www.nfid.org/conferences/vaccine20/absposter). Also, The CpG is found to have the ability to induce an innate immune response in fish later challenged with the furunculosis causing bacteria *A. salmonicida* (Carrington *et al*., 2002). However, few studies have been carried out on CpG ODN in fish, these studies point to the immunological effects of CpG-ODN and the possibility of using it as adjuvant in fish vaccines. In addition, there is no evidence of negative side-effects or toxicity (Weeratna *et al*., 2000).

Adjuvants containing aluminium compounds are approved adjuvants used in human practice. Aluminium hydroxide has been used in rainbow trout but showed no effect (Horne *et al*., 1984). Another aluminium adjuvant is potassium aluminium sulphate used in vibrio vaccine.

1.5.6 Vaccination against atypical and typical furunculosis

Studies of the bacterium and vaccination against typical or classical furunculosis have been carried out in salmonids and non-salmonid fish species for more than a 100 years (Bernoth *et al*., 1997). However, few studies are carried out on Atlantic cod.

Vaccination of salmon by oil-based vaccines against *A. salmonicida* protects the fish against furunclosis and also accompanied by an elevation of specific antibodies even at low temperature (Eggset *et al*., 1997: Madtlyng, 1997).

According to Bernoth *et al*., (1997) the importance of furunculosis disease has been related to the importance of salmonids fish species and its impact on this industry. In addition, Murno and Hasting (1993) indicate the significant of furunculosis. Therefore, numerous investigations have been done in order to control the disease by vaccination. Furthermore, studies on pathogenicity of the causative bacteria, bacterins protective immunity and comparison of different types of vaccines (Thronton *et al*., 1994; Thronton, 1995). All these efforts of research have been done give useful results and information, which leads to the present developments in vaccination protection and effectiveness of the commercially available vaccines against typical and atypical furunculosis (Peterson *et al*., 1985; Cardella and Eimers, 1990; Lund *et al*., 2002a; Midtlyng, 2000).

2. AIM OF THIS STUDY

2.1 The Research Problem

The use of injectable vaccines included oil adjuvants in salmonids and non-salmonids species leads to undesirable intra-abdominal lesions, known as side-effects. These side effects seem to be even more pronounced in Atlantic cod compared to salmonids (M. Schrøder, pers.comm.).

2.2 Objective of the study

The main objectives of this study are:

1. Compare several adjuvant combinations and their ability to induce protection against atypical furunculois in Atlantic cod.
2. Investigate the side effects of the adjuvants presented in this study, which are CpG DNA, Freunds Incomplete Adjuvants (FIA), and Aluminium Hydroxide (Alhydragel).
3. Evaluate the antibody responses after vaccination of Atlantic cod against atypical furunculosis.

2.3 Significance of this study

The finding of this study will increase the existing knowledge of immunoprophylaxis in Atlantic cod against atypical *A.salmonicida*. Furthermore, the findings are also expected to assist vaccine industry and interested companies to make effective anti-atypical furunculosis vaccines for Atlantic cod.

2.4 Thesis out lines

The first section of this thesis is a general introduction to the topic emphasising the biology, and background of vaccination against fish diseases. The second chapter is the aim of the presents study, the research problems, and the main objectives of the present study will be are presented.

The third chapter describes the material and methods used in this study, including experimental design and procedure. The data collected will be used to calculate the relative percent survival (RPS) using special formula (see material and method), and these are presented in the result chapter, i.e. the fourth chapter. In addition, all the

statistical analysis results will be presented in this chapter. The fifth chapter discusses the results of the entire study with respect to the efficacy of the vaccines and the side-effects of the different adjuvants. The conclusion is presented in the final chapter, which is chapter 6.

2.5 Limitation of the present study

Based on the fact that vaccine effect should be drawn based on the results of various biological parameters and environmental factors, the present study is limited in certain issues due to different situations and factors. The present vaccines are used on small Atlantic cod under experimental conditions. The time limits further investigation of the protection and side-effects after 12 weeks. Other factors that could have been evaluated are the effect of the adjuvants on other pathogens (as virus) and the protection obtained at different water temperatures.

3. MATERIALS AND METHODS

This section provides detailed information about the material and methods used to run this experimental study. A vaccination trial including one vaccination and one challenge experiment was performed at the aquaculture research station and the fish health laboratory (FHL), Kårvika, Tromsø, Norway.

3.1. Experimental design

Atlantic cod (*Gadus morhua* L.) were intensively reared and obtained from a local hatchery (Troms Marin Yngel). The fish were 1 g when transferred to the aquaculture research station. The fish were reared in seawater at 10°C, fed on a commercial pellet diet, and kept at the experimental unit of the station (L.A) during the experiment. When the fish was approximately 35 g they were placed in 10 tanks with 90 fish in each tank (300L) as described in Table 3.1.

The fish anaesthetised with metomidate (10mg/L) and vaccinated by intraperitoneal injection (i.p.) of 0,1 ml of different vaccines as described in Table 3.2.

Prior to challenge the fish were anaesthetised with metomidate (10mg/L) and marked with fluorescent tags (Northwest marine technology, inc.) as shown in appendix E. Individual weights of 20 fish from each group were taken prior to both vaccination and challenge.

For the challenge experiment, 80 fish were transferred to FHL and distributed into two tanks (900L). The fish were intraperitoneally (i.p) infected with atypical *A. salmonicida* and mortality was registered daily for 43 days. The cause of death was confirmed by seeding samples from the head kidney of moribund fish on blood agar plates for reisolation of bacteria and incubated at 12°C for 24 hours. *A. salmonicida* was confirmed by the use of Mono-As kit (Bio-Nor). Intraabdominal lesions were recorded in 20 fish per group. Eight fish from each group was left in one tank (300L), for three more weeks post challenge, in order to be used for the blood samples.

Table 3.1 Vaccination experiment design of Atlantic cod: groups, antigen, adjuvant and number of fish in each group. (0.9% NaCl).

Group No.	Tank No.	Bacterial Antigen	Adjuvants	No. of fishes
1	1	*A.salmonicida* (atypical)	CpG FiA	90
2	2	*A.salmonicida* (atypical)	CpG	90
3	3	*A.salmonicida* (atypical)	FiA	90
4	4	*A.salmonicida* (atypical)	CpG+Alum	90
5	5	*A.salmonicida* (atypical)	-	90
6	6	NaCl	CpG FiA	90
7	7	NaCl	CpG	90
8	8	NaCl	FiA	90
9	9	NaCl	CpG+Alum	90
10	10	NaCl	-	90

3.2. Vaccines

3.2.1 Bacteria

Atypical *A. salmonicida* (aAs 4099), originally isolated from cod in Iceland, was used for vaccination and challenge of Atlantic cod.

The bacteria were transferred from glucerol-culture (- 80° C) and seeded on blood agar supplemented with 2% NaCl and grown at 12°C for 72 hours. One colony was transferred to 10 ml of brain heart infusion broth (BHI, Difco) and incubated on an orbital shaker at 12°C for 24- 48 hours with shaking.

The bacterial cells used in the vaccines were inactivated over night after addition of formalin 0.5% (v/v), centrifuged (Sorvall SS- 34 at 4000 rpm for 15 minutes) and diluted in 2% NaCl to a final concentration of $4x\ 10^9$ bacteria/ml ($OD_{600} = 4$). Inactivation was confirmed by seeding the suspension on blood agar with 2% NaCl and incubated at 12° C for 72 hours.

3.2.2 Preparation of vaccines

The vaccines consist of different adjuvants and atypical *A. salmonicida* bacterin as explained in Table 3.2. The adjuvants used were CpG ODN 2006 (5 mg/ml) with the sequence of 24 bases (5´TCGTCGTTTTGTCGTTTTGTCGTT3´) (OliGold). This manufacture was chosen because they offered the cheapest CpG . However, the extract was in some extend more crude than CpG offered from other producers (J. Jørgensen, pers. Comm.). The other adjuvants are Freunds incomplete adjuvant (FIA, Sigma) and Aluminium hydroxide gel (Alhydrogel). The bacteria were atypical *A. salmonicida* (aAs 4099). The bacterial stock described above was diluted in order to obtain appropriate cell concentration in the different vaccines and added to adjuvants (Table 3.3). In vaccine number 1, 3, 6, and 8 the antigen were added to the oil during heavily mixing and the vaccines were then stored at 4^{o} C for 24 hours. In the other vaccines the antigen and adjuvants were normally mixed.

3.3. Vaccination

The fish were anaesthetised with metomidate (10 mg/L) and individually vaccinated by i.p. of 0.1 ml of the vaccines using 1 ml syringes. The vaccines are described in Table 3.2. The concentrations of the components in the vaccines are shown in Table 3.3. After vaccination the fish were kept in 10 tanks (300L) with 90 fish in each group as shown in Table 3.1. The fish were fed a commercial pellet diet through out the experiment. The water temperature was registered daily (appendix F). Twenty fish from each group were weighted at the time of vaccination.

3.4 Prechallenge experiment

In order to determine the bacterial dose, which will be used in the challenge experiment for the vaccinated groups, a prechallenge experiment was carried out. The prechallenge experiment consists of 3 groups of cod (30 fish/group) were injected intramuscular (i.m.) with atypical *A.salmonicida* strain aAs 4099. The bacterial doses used were 6×10^3, 6×10^4, and 6×10^5 bacteria/fish. The strain was transferred from glycerol-culture (-80º C) planted on blood agar with 2%NaCl and incubated at 12ºC for 48 hours. Pure colonies were transferred to brain heart infusion broth (10 ml) and incubated with shaking over night at 12ºC. The bacterial cells were collected by centrifugation at 13000 rpm for 5 minutes and resuspended in 0.9% NaCl. The fish

weighted 100 g and the i.m. injection was done on the lateral side of the fish body between the second dorsal fin and the lateral line.

Table 3.2 Different vaccine formulations used to vaccinate Atlantic cod. The bacteria are atypical *A.salmonicida* (aAs 4099) with or without CPG DNA, Freunds incomplete adjuvant (FIA), Aluminium hydroxide (Alhydrogel) and CpG in combination with Aluminium .

Vaccine No.	Bacteria OD_{600}=4	CpG 2006	Freunds Incomplate Adjuvans	0,9% NaCl	Alhydrogel® 13 mg/ml	Total volume
1	5 ml	2 ml	10 ml	3 ml	-	20 ml
2	3 ml	1,2 ml	-	7,8	-	12 ml
3	5 ml	-	10 ml	5 ml	-	20 ml
4	$1,2x10^{10}$	6 mg	-	-	12 ml	12 ml
5	5 ml	-	-	15 ml	-	20 ml
6	-	2 ml	10 ml	8 ml	-	20 ml
7	-	1,2 ml	-	10,8 ml	-	12 ml
8	-	-	10ml	10ml	-	20 ml
9	-	6 mg	-	-	12 ml	12 ml
10	-	-	-	20ml	-	20 ml

Table 3.3 The concentration of the different components of the vaccines. The bacteria are atypical *A.salmonicida* (aAs 4099), in CpG DNA,or FIA in combination.

Components	Concentration in vaccines	Concentration in fish
Bacteria	10^9 bacteria/ml	10^8 bacteria/fish
CpG (2006)	500 μg/ml	50 μg/fish
$Al(OH)_3$	13 mg/ml	1.3 mg/fish
FIA	1:1	-

3.5 Tagging system of the fish

Approximately one month before the challenge, the fish were anaesthetized with metomidate (10mg/L) and individually tagged with fluorescent tags (Northwest marine technology, inc.) by subcutaneous injection near the operculum. The tagging system is shown in appendix E.

3.6 Challenge experiment of the Atlantic cod

Twelve weeks post vaccination, the challenge was performed with the same atypical *A. salmonicida* strain as used in the vaccination. Eighty fish from each group were transported to the fish health laboratory (FHL) anaesthetised with metomidate (10mg/L) and i.p. injected with 0.1 ml of atypical *A. salmonicida*. The fish were not intramuscularly (i.m.) infected because the prechallenge resulted in very heavy mortality in all groups. Due to this result the intraperitoneal (i.p.) infection was chosen. The challenge dose used was 5×10^5 bacteria / fish. This dose was determined dependent on a previous prechallenge experiment. The fish were then redistributed in two tanks (900L), approximately 40 fish from each group in each tank. Mortality in each group was recorded daily. Cumulative mortality was registered and relative percent survival (RPS) was recorded at the end of the experiment (day 43 after challenge), according to the formula:

RPS=(1-%mortality in vaccinated group/mortality in control group) X 100%

3.7 Evaluation of the intraabdominal lesion

Dead fish, 20 from each group, were examined for side-effects. The dead fish were shipped on ice to the laboratory for autopsy the same or the following day. Each fish was investigated by a ventral incision of the abdominal cavity and description of the visible intraabdominal lesion and adhesions was recorded. According to the visual appearance of the abdominal cavity and the severity of the lesions, each fish was given a score from zero to three. The score 0= no visible lesions; the score 1= injection site adhesions; and the score 2= adhesions between the internal organs by non-transparent membrane and liver attached to the internal organ and the abdominal wall.

Some non-infected fish were inspected for side-effects in order to confirm that side-effects observed in the diseased fish were caused by the vaccine and not by the infection

3.8 Blood sampling

Eight fish from each group were used to collect blood samples for evaluation of the post vaccination antibody level fifteen weeks post vaccination. The fish was anaesthetised (metomidate10mg/L) and blood was sampled from the caudal vein using vacuum tubes and injectable syringes. The blood samples were stored on ice over night and centrifuged at 1000 rpm for 10 minutes in order to separate the serum from the blood. The sera were stored at – 20°C until use.

3.9 ELISA with atypical *A. salmonicida* (aA.s.) in Atlantic cod

The antibody responses in cod to atypical A. salmoncida were measured by Enzyme-linked Immunosorbent Assay (ELISA) in pooled and individual sera. This test is done as described by (Magnadottir *et al*., 1998c) with some modification.

Solutions used during the ELISA.

Phosphate buffered saline (PBS)

10X:

220.6 g Na_2 HPO_4*$12H_2O$

28.74 g Na H_2 PO4*$_2H_2O$

350.4 g NaCl

4 L of distilled water.

pH 7.3

Carbonate buffer

1.59 g $NaCO_3$

2.93 g $NaHCO_3$

1 L distilled water (H_2O).

pH 9.6

2% saline (2% NaCl)

20 g NaCl.

1 litter distilled water (H_2O)

Washing buffer: 1 x PBS with 0.05% Tween 20[1]

For making 3 Litres:

300 ml 10 x PBS

2700 ml H_20

1.5 ml Tween 20 (Sigma).

pH 7.4

5% Dry skimmed milk in PBS

10 ml 10 x PBS

90 ml distilled H_2O.

5 g of dry skim milk.

pH adjusted to 7.4

0.5% Dry skim milk in PBS

10 ml 10 x PBS

90 ml distilled H_2O.

0.5 g dry skim milk.

pH adjusted the to 7.4

Poly-L-Lysin (5mg/ml)

A stock solution of Poly-L-Lysin in water (5mg/ml, Sigma) was diluted 1:1000 in carbonate buffer.

Substrate Buffer

97 ml Diethanolamine

900 ml distilled H_2O

100 $MgCl_2$ x $6H_2O$ (1mg/ml)

pH 9.6

The volume was adjusted to 1 litre.

ELISA was performed in 96-wells polystyrene plates (Nunc). The plates were coated with poly-L-Lysin (5µg/ml) in carbonate buffer (100 µl per well) and incubated overnight at 4° C. Then the plates were washed with washing buffer and kept at 4° C. Atypical *A. salmonicida*, isolated from the head kidney from the dead fish, were cultured on blood agar at 12°C for 24 hours, recultured on blood agar plates

[1] Tween 20 chemically is Polyoxethylene-sorbitan monolaurate

(supplemented with 2% NaCl), and incubated at 12°C for 3 days. One bacteria colony was transferred from the blood agar plate to 5 ml of BHI broth media (18.5 g Heart Brain Infusion in 500ml distilled water and autoclaved) and incubated at 12°C for 24 hours. 1 ml of this cultured bacterium was added to 200 ml BHI broth media, and incubated at 12°C for 24 hours.

The cultured bacteria was fixed by adding 200 µl of formalin to 40 ml bacteria (final concentration of 0.5% v/v) and then incubated over night at 4° C.

The fixed bacteria were centrifuged at 3500 rpm for 15 minutes, at 20° C. The sediment was then diluted in 2% NaCl (1:20). Optical density was measured using photometry. OD_{600} was 0.410, which means (4x 10^8 bacteria/ml). This bacterial solution was used to coat the wells of the microtitre plates with 100 µl bacteria per well. The plates were centrifuged at 1000 rpm for 10 minutes at 4° C and incubated for 30 minutes at room temperature (20°C) in order to coat the bacteria to the wells of the microtitre plates the plates. The plates were washed 3 times with the washing buffer. The plates were blocked with 125 µl 5% skimmed milk in PBS per well and incubated at 4°C over night. The plates were washed 3 times with the washing buffer and stored at – 20°C until used.

The bacteria coated to the microtitre plates were used to measure the antibody level in pooled and individual cod serum. The samples of cod serum were pooled and diluted 1: 100 by adding 5-10 µl pooled serum to 500 –1000 µl of 0.5% Dry skimmed milk. Then the sera were two fold diluted from 1: 100 to 1:6400 in two parallel wells and incubated at 4°C over night. At this stage, specific cod immunoglobulins will bind to the bacteria coated to the microtitre plates.

The plates were then washed 3 times with the washing buffer and a rabbit anti-cod serum (M. B. Schrøder) were diluted 1:800 d in 0.5% (w/v) skimmed milk in PBS, was added to the microtiter plates (100 µl per well). The plates were incubated for 2 hours at room temperature (20°C).

The plates were washed 3 times with the washing buffer and goat anti-rabbit immunoglobulins conjugated with alkaline phosphate (sigma) diluted 1:30000 in 0.5% w/v skimmed milk, was added (100µl per well). The plates were incubated at room temperature (20°C) for one and a half hour.

Finally, after washing 3 times with the washing buffer, 100µl per well of p-nitrofenyl phosphate substrate (Sigma) in substrate buffer (1mg/ml) was added to all the wells. The plates were incubated in dark for 30- 45 minutes at room temperature (20^0C) and the optical density was read at 405 nm using "Thermo max microplate reader" Version 4.0 Software, (SOFTmax PRO User's Manual Chapter 4).

3.10 Statistical Method of Analysis

Computerised statistical techniques were used to analyse the results from the primary data. A Yates corrected Chi-square test (χ^2) was used to determine statistical differences in mortality between vaccinated groups and unvaccinated group using Ms-Excel programme.

Analysis of variance (ANOVA) was used to analyse the ELISA results and also to determine the statistical differences in weights between vaccinated groups (Berk and Carey, (2000), chapter 10). The results will be considered significant if $p < 0.05$.

4. RESULTS

4.1 Prechallenge

The intramuscular (i.m.) prechallenge of Atlantic cod with atypical *A.salmonicida* resulted in high mortality and all the fish die within 10 days (Fig. 4.1). Because of this result the challenge experiment was decided to be with intraperitoneal injection (i.p.) and the doses used were based on experience from a previous prechallenge, where $1.5x10^5$ bacteria per fish gave 35% mortality. We therefore decided to use $5x10^5$ bacteria per fish administered i.p. in this present study.

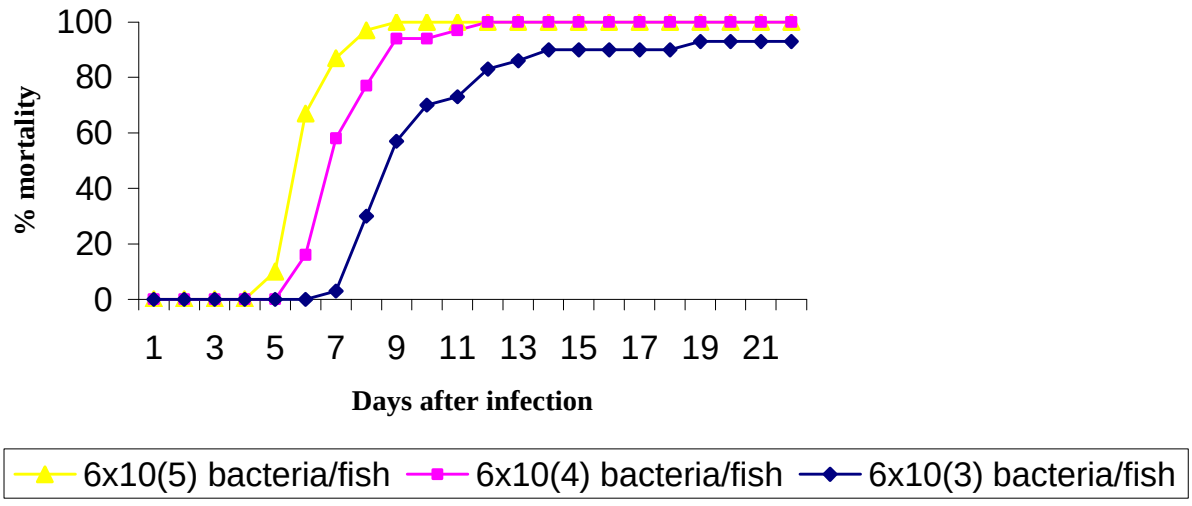


Figure 4.1 Intramuscular (i.m.) infection of Atlantic cod with atypical *A. salmonicida* strain aAs 4099 using three doses $6x10^5$, $6x10^4$, and $6x10^3$ bacterial/fish.

4.2 Vaccination and challenge

Ten groups of Atlantic cod were vaccinated with different vaccine formulations using atypical *A.salmonicida* bacterin and FIA, CpG, and Aluminium hydroxide adjuvants. Approximately eighty fish in each group, equally distributed in two tanks, were challenged 12 weeks post vaccination. Fourty-three days post challenge there were no significant differences in mortality between each group in the parallel tanks and the results from the two tanks are thus presented together.

The same atypical *A. salmonicida* strain (aAs 4099) was used both in the vaccines in the prechallenge and in the challenge experiment. All the vaccinated groups were infected with a dose of $5x10^5$ bacteria/fish (0.1ml).

The results from the challenge experiment with atypical *A. salmonicida* are presented as cumulative mortality in Fig 4.2.

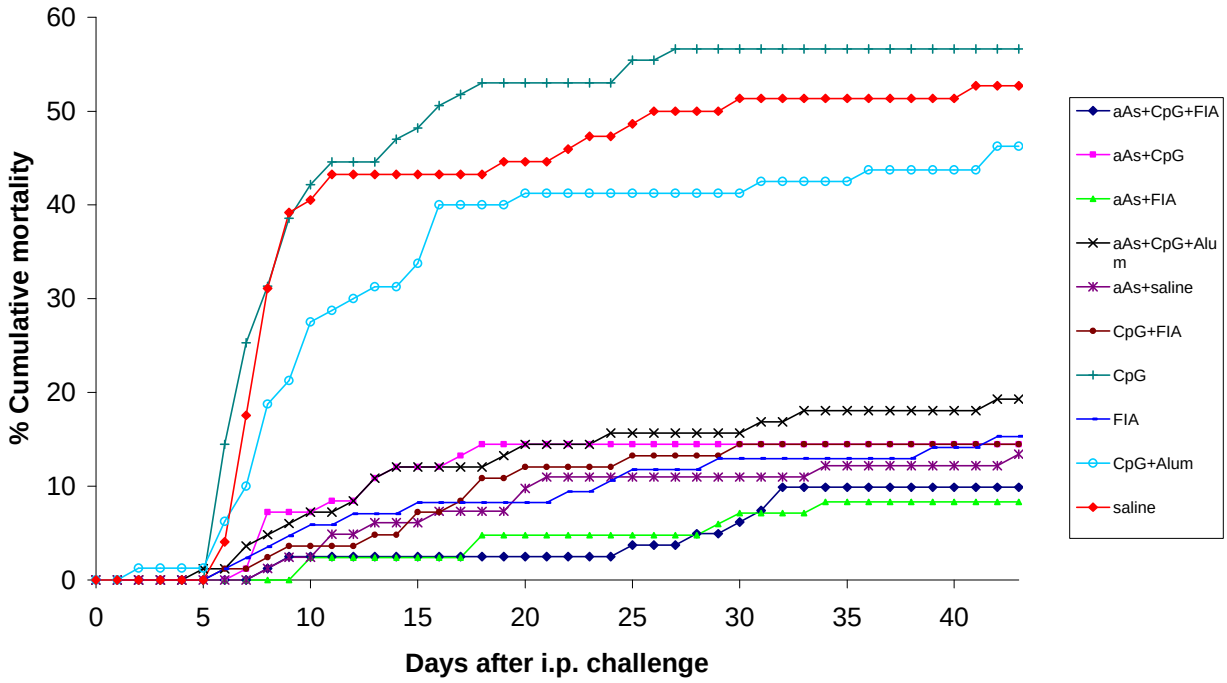


Figure 4.2 Atlantic cod vaccinated (i.p.) with atypical *A.salmonicida* (aAs 4099) bacterin alone or in combination either with CpG DNA, Freunds incomplete adjuvants (FIA), CpG and Aluminium Hydroxide (Alum) or CpG and FIA. Control groups received the respective adjuvants alone. The fish were challenged (i.p.) 12 weeks post vaccination with atypical *A.salmonicida* aAs 4099 (5×10^5 bacteria/fish).

The fish in groups 7 (CpG), group 9 (CpG+Alum), and control group started to die at day 5 post challenge. During the next 10 days the mortality in these groups increased rapidly and reached 40-50%. The mortality in the other groups did not exceed 20% during the experiment. In group 3 (aAs+FIA) the fish started to die at day 9 after challenge and the mortality remain low (8%) throughout the infection. At the end of the experiment the mortality was declined in all groups. The mortality reached 53% in the saline control group, while the mortality in the group 7, (CpG alone), was 57 %. The vaccine with bacterin alone resulted in low mortality (13%) and the bacterin with FIA resulted in even lower but not significantly, mortality.

The vaccine CpG+ Alum without antigen resulted in 46% mortality, whereas when the antigen was included, the mortality was only 19%.

The efficacy of the vaccines is calculated as relative percent survival (RPS) on day 43 post challenge and the results are presented in Table 4.1 and appendix A.

Table 4.1 Dead, and Survival Atlantic cod and vaccines efficacy (RPS), in the different groups (43 days post challenge).

Group No	Vaccine type	Challenge strain aAs 4099			
		Dead	Survived	Mortality (%)	RPS (%)
1	aAs+CpG+FIA	8	73	10	81
2	aAs+CpG	12	71	14	73
3	aAs+FIA	7	77	8	84
4	aAs+CpG+Alum	16	67	19	63
5	aAs+saline	11	71	13	75
6	CpG+FIA	12	71	14	73
7	CpG	47	36	57	-7*
8	FIA	13	72	15	71
9	CpG+Alum	37	43	46	12
10	Saline	39	35	53	0

* Negative due to the higher cumulative mortality% in group 7 (57%) compared to control group (53%).

Lower mortality was observed in the groups vaccinated with oil-based vaccines compared to the control group, even when the fish did not receive antigen. This give a RPS of 81%, 84%, 73%, and 71% in group 1 (aAs+CpG+FIA), group 3 (aAs+FIA), group 6 (CpG+FIA), and group 8 (FIA) compared to the control group. In addition, the mortality in group 5 (aAs in saline) was 13% and the RPS 75%. This indicates that bacterin alone can give similar protection as the FIA-emulsified vaccines in Atlantic cod, at least during a limited period of time. Further more, the RPS in group 2 (aAs +CpG) 73%, which is similar to the mortality in group 5 (aAs+saline). Since the protection after vaccination with antigen alone gave such a low mortality, it is not possible to make any conclusions about the effect of CpG on the protection in cod. Also, CpG alone gave the highest mortality (57 %), which gives a negative RPS value. Furthermore, a low protection level was obtained when antigen was combined with Alum (RPS= 63%).

Among the vaccinated groups, the differences were tested by mean of χ^2-test analysis. The mortality in the vaccinated groups were compared with the control group, with each other, or with their counterparts (group received the same adjuvants without antigen) and the results are shown in appendix C. The results show that vaccines containing antigen gave a higher protection than the control group ($p< 0.001$). The bacterin alone gave significant protection compared to the control group. In the groups which received CpG alone or CpG+ Alum the protection level is not significant different from the control group.

Comparisons of the groups which received aAs bacterin with adjuvants and their contourparts, showed no significant differences between groups received aAs+FIA or aAs+CpG+FIA, whereas there is significant difference between groups received aAs+ CpG or aAs+CpG+Alum and their counterparts. This confirms that FIA alone give a high non-specific protection in cod (appendix C-2).

No significant differences in protection between groups received antigen with each other ($p> 0.05$), except (aAs+FIA) and (aAs+CpG+Alum) show small difference ($p< 0.05$) as shown in appendix C-3.

4.3 Antibody responses to atypical *A. salmonicida*

Sera from all the vaccinated groups were collected 15 weeks post vaccination. The antibody responses were measured in pooled serum from all the vaccinated groups and individual sera from group 1 (aAs+CpG+FIA), group 3 (aAs+FIA), group 6 (CpG+FIA), and group 8 (FIA). The results showed an increased antibody activity to whole cells of atypical *A. salmonicida* aAs 4099 in pooled sera (Fig.4.3 and appendix B). Cod immunised with atypical *A. salmonicida* bacterin in saline alone or in combination with FIA showed high antibody responses, while the other vaccines gave low levels of antibody responses. There is significant difference in the antibody responses between the vaccinated groups and unvaccinated groups (One way ANOVA, p-value 0.00002).

Results from the individual sera antibody responses are presented in figures 4.5 and 4.6. The fish were either immunised with aAs+CpG+FIA (group 1) or aAs+FIA (group (3) or CpG+FIA (group 6) or FIA alone (group 8). The antibody responses are higher in the vaccines containing the antigen compared to vaccines without the antigen.

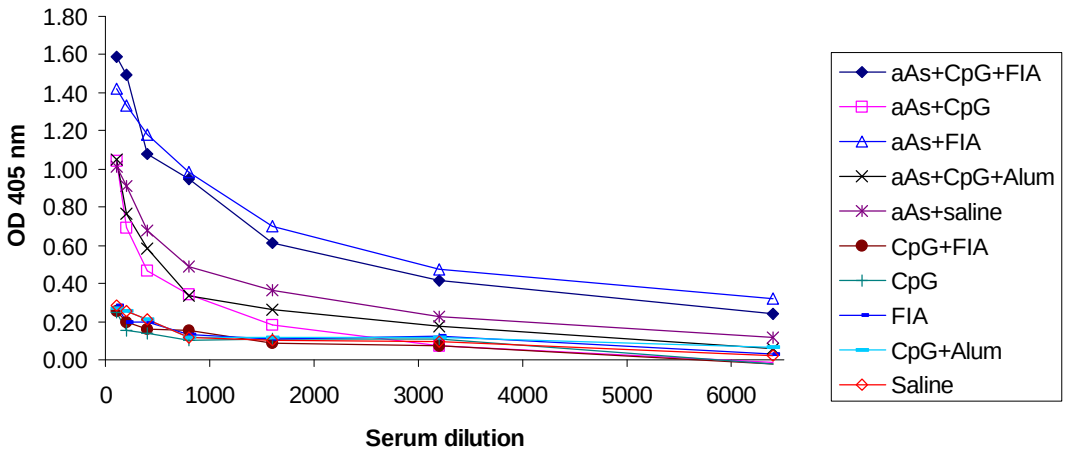


Figure 4.3 Antibody activity in sera from Atlantic cod vaccinated with different vaccines against atypical *A. salmonicida*. The sera were pooled (n=8) and tested against *A. salmonicida* whole cell in ELISA. Diluted 1:6400, 15 weeks post vaccination.

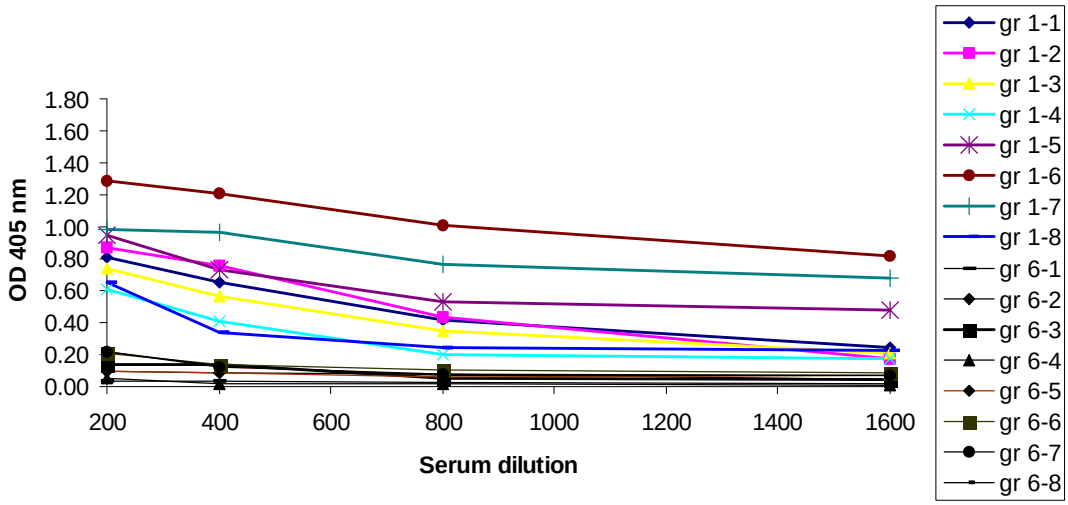


Figure 4.4 Antibody activities in individual sera from Atlantic cod measured against whole cells of atypical *A. salmonicida* (aAs 4099) in ELISA (n=8). The fish were either immunised with aAs+CpG+FIA (group 1) or CpG+FIA (group 6). Each line represents one individual.

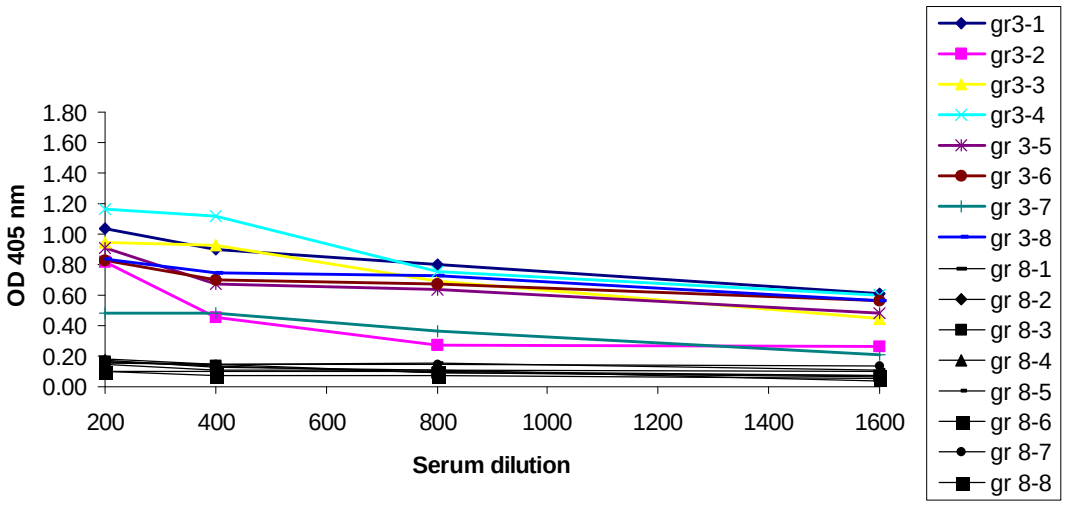


Figure 4.5 Antibody activities in individual sera from Atlantic cod measured against atypical *A. salmonicida* (aAs 4099) in ELISA (n=8). The fish were either immunised with aAs+ FIA (group 3) or FIA alone (group 8). Each line represents one individual.

4.4 Vaccine side-effects

Twenty fish from each group were inspected in order to evaluate the side-effect of the different vaccines. The lesions were given a score from 0 to 2 (Table 4.2). The observed side-effects in the this study is shown in figure 4.7.

The side-effects were visible in groups of fish vaccinated with FIA and in groups vaccinated with Aluminium hydroxide.

The FIA led to sever intraperitoneal adhesions. The internal organs were firmly attached to the abdominal wall and surrounded by non-transparent membranes (Fig. 4.7 d & e). The non-transparent membranes contained the vaccine droplets. Visible adhesions between the liver and the abdominal wall were observed in all the investigated fish from these vaccines (group 1, 3, 6 and 8). These side-effects were evaluated as score 2. Also, it was found that side-effects were presented in the same degree in all the investigated fish treated with FIA.

The fish which received vaccines containing Aluminium hydroxide showed inflammation and adhesions between the abdominal wall and the internal organ close to the site of injection. No adhesions were observed elsewhere in the body cavity (Fig.7.4c). These side-effects were evaluated as score 1.

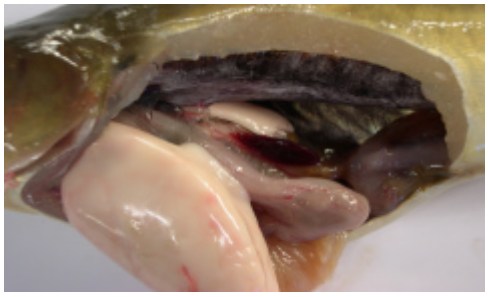
No side-effects were observed in unvaccinated fish, or in fish which received bacterin or CpG alone or in combination. This finding was evaluated as score 0.

There were no significant differences between the non-infected and infected fish. Thus, the lesions observed were caused by the treatment with the vaccines and not the infection.

Table 4.2 Score and intra-abdominal lesions in different vaccinated groups of Atlantic cod (*Gadus morhua* L.) 12 - 15 weeks post vaccination.

Vaccines	Group No	Score	Intra-abdominal lesions
aAs+CpG	2	0	
aAs+saline	5		No visible lesions
CpG	7		
Saline	10		
aAs+CpG+Alum	4	1	Adhesion at the injection site
CpG+Alum	9		
aAs+CpG+FIA	1	2	Liver severely attached to abdominal
aAs+FIA	3		wall and connected with internal
CpG+FIA	6		organs by non-transparent membrane
FIA	8		containing vaccine droplets.

a. Unvaccinated normal internal organs**.**



b. Score 0: No side-effects.

c. Score 1: the liver, the abdominal wall and the internal organs adhesion at the injection site

d. Score 2: Liver attached to the abdominal

e. Score 2: Non transparent membranes between the internal organs. The organs are firmly attached to each other.

Figure 4.6 Side-effects lesion and scores in Atlantic cod vaccinated against atypical furunculosis using FIA, CpG DNA and Aluminium hydroxide. **a**.Unvaccinated fish normal internal organs. **b** . Side-effect of CpG adjuvaned vaccines. **c**. Side-effect of Alum adjuvanted vaccines. **d** & **e**: Side-effects of FIA adjuvanted vaccines.

The fish were weighted at the time of vaccination and 12 weeks post vaccination (figure 4.8, and appendix D). Before the vaccination, there was no significant difference in the weights between the fish groups (one way ANOVA, p=0.2515) wheras12 weeks post vaccination it was found a significant differences between the groups weights in particularly the group vaccinated with aAs+CpG+Alum vaccine (p-value 0.00036).

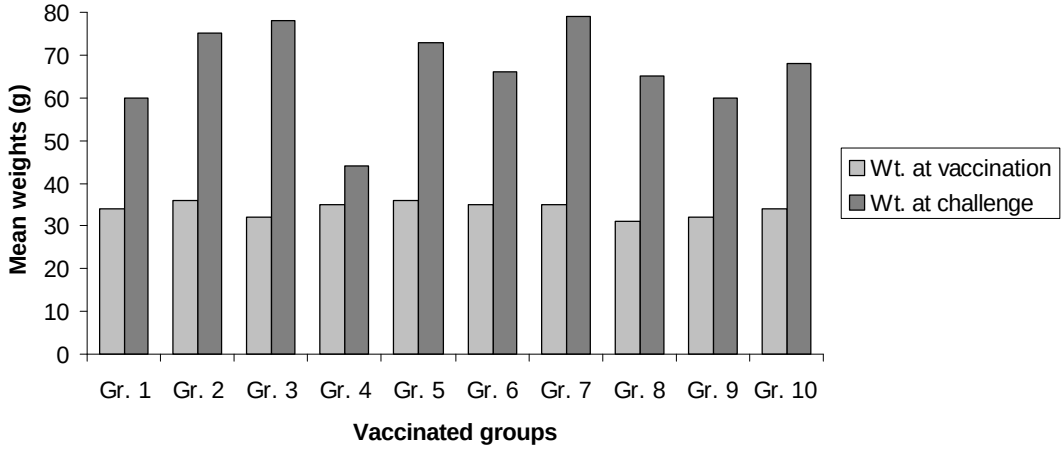


Figure 4.7 Mean weights of Atlantic cod before and after vaccination with the different vaccines against atypical furunculosis.

5. DISCUSSION

5.1 Protection post vaccination

In the presented work all vaccines containing antigen protected cod highly against atypical furunculosis compared to the saline group and no significant differences were observed between the groups, which received antigen in different adjuvants. This means that addition of adjuvants did not improve the protection significantly, even if Freunds incomplete adjuvant (FIA) was used. In contrast to this finding, furunculosis vaccines became protective in salmon when mineral oil adjuuvants were added (Lillehaug *et al*., 1992; Midtlyng *et al*., 1996a; Midtlyng *et al*.1996b; Gudding *et al*., 1999; Midtlyng, 2000). Also, injection of formalin inactiviated *A. salmonicida* without adjuvant in brown trout (*Salmo trutta*) induces a low protection (Krantz *et al*., 1964). Furthermore, vaccination of spotted wolffish and halibut with oil adjuvanted vaccines against atypical furunculosis gives good protection.

Fish injected with FIA alone obtained a high non-specific protection (RPS=71). This result is in line with previous findings when vaccinating cod against classical vibriosis (Schrøder pers. comm.). Generally, oil-based vaccines are well known to afford long-term protection against different antigens (Bowden *et al*., 2003). Their mode of action is thought to be in large part due to stimulation of non-specific defence mechanisms by low spot release, which can occur in the absence of the vaccine antigen (Oliver *et al*., 1985b; Adams *et al*., 1988) but exactly how this relates to protection is not yet known. Also other adjuvants enhance the protection after vaccination.

The protection obtained in cod, which received antigen in CpG with Aluminium hydroxide and CpG and Aluminium hydroxide was relatively low compared to other groups received adjuvanted *A. salmonicida* bacterin. The mortality in the groups receiving CpG alone or in combination with Aluminium Hydroxide was similar to the saline control group. This may be due to the combination with CpG adjuvant.

In consistency with previous reports in salmonids, vaccination with aluminium hydroxide adjuvanted bacterins resulted in poor protection of Atlantic cod against atypical furunculosis.

This revealed the role of the antigen in the induction of the protective immunity and that the adjuvant alone is not enough for effective protection.

The CpG in this study gives protection when used in combination with antigen (aAs).

However, because of the high protection when vaccinating cod with bacterin alone no conclusion can be drawn about the effect of using CpG as an adjuvant in cod. The high mortality obtained from the CpG alone may be is a result of its crudeness. CpG is known as an adjuvant, which stimulates immune responses in fish through its enhancement of antibodies, stimulation of macrophages and production of cytokines, such as gamma interferon (IFN-γ) and IL-Iβ, (Jørgensen *et al*., 2001; Jørgensen *et al*., 2002). In murine models, studies of CpG have been reported to activate B cells, macrophage, natural killer cells and even more cell types (Mutwiri *et al*., 2003; Harandi *et al*., 2003). However, details of the mechanisms that mediate protection are not fully understood, this is due to the variety of cells activated as well as the spacing between CpG motifs, which may influence the type of immune stimulation (Mutwiri *et al*., 2003). Furthermore, the sequence and the number of CpG motifs affect the stimulatory activity in Atlantic salmon (Jørgensen *et al*., 2003). This fact may contribute to the poor protection of CpG in cod and different CpG motifs should be tested in cod as in salmon.

The susceptibility of cod to atypical *A. salmonicida* appears to be high as infected cod start to die without showing any of the disease symptoms when infected i.m. or i.p. with *A. salmonicida* (Cornick *et al*., 1984; Magnadottir *et al*., 2002). In this study vaccination and infection was both done i.p. due to the high mortality with i.m. infection. However, after more than one month the diseased fish showed the skin ulceration and furuncle in different parts of the body. Since atypical *A. salmonicida* strains are genetically diverse according to Lund *et al*., 2002b, further test to the potency of *A. salmonicida* bacterin using different strains for challenge is recommended.

5.2 Antibody response post vaccination

In this study, antibody responses to atypical *A. salmonicida* aAs-99 whole cells, both in pooled and individual sera were measured. Surprisingly, specific antibodies responses were demonstrated in all groups vaccinated with atypical *A. salmonicida.* The responses were strongest in groups receiving bacterin in FIA, whereas, the level of specific antibodies were weaker in fish vaccinated with *A. salmonicida* in saline. The bacterin in CpG alone or in combination with Aluminium hydroxide was least effective for augmenting antibody responses.This finiding contradict with previous

reports, which showed that the CpG as an adjuvant, stronger immune response against a model antigen in mice was obtained compared to FIA adjuvant (Weeratna *et al*., 2000; Harandi *et al*., 2003).

The antibody responses in individual sera varied in the level but followed the same pattern of responses as the pooled sera. These results indicate that cod can produce a significant level of specific antibody response after vaccination against atypical furunculosis. Previous work in cod has shown that cod do not produce specific antibodies after immunisation with *V. salmonicida* or protein antigens (Schrøder *et al*., 1992; Pilström and Petersson, 1991) Immunisation with *V. anguillarum*, however, have resulted in weak responses (Espelid *et al*., 1991).

Except from this information studies of the immune system of cod has not shown any specific features and this indicate that there are no clear differences between the cod immune system and the immune system in other fish species (Magnadóttir *et al*., 2001: Stenvik 2001). Also, the antibodies obtained in this study confirm that the cod immune system does not deviate from other species and that the lack of responses might be related to the nature of the antigen.

Moreover, the results in this study showed that the specific immune responses against atypical *A. salmonicida* were correlated with the protection. The antibody levels and the magnitude of protection were consistency positively associated, as the highest responses were observed in the groups, which were best protected in the challenge. Most studies on the relation between immune response and protection have been performed with injectable vaccines. In previous studies with *A. salmonicid,* protection in salmonids was found related to the serum antibody titre (Bricknell *et al*., 1997; Midtlyng *et al*., 1996a; McCarthy *et al*., 1998). The production of antibodies, resulting from the proliferation of primary effector's cells and their differentiation into both antibody secreting cells and specific memory cells, is often a complex process requiring cell cooperation. This is important, since the magnitude of these responses will reflect the ability of different vaccines to evoke memory responses to atypical *A. salmonicida*. It is necessary to produce a strong memory response for a sustained duration, otherwise antibody titres and cell-mediated response will decline. Thus, to be able to develop new, cheap and effective vaccines against atypical furunculosis for the growing Atlantic cod aquaculture industry more research should focus on the immune system especially the cellular immune system.

Further studies have been done on the sera obtained in this study using western blotting techniques. The results indicate that the specific antibodies mainly directed against the bacterial lipopolysaccharide (LPS) and to less extend to the A-layer (V. Lund pers. communication). These findings are not inconsistence with previous results in salmon, spotted wolffish and Halibut where immunisation with atypical *A. salmonicida,* resulted in a strong immune response against the A-layer (Lund *et al.*, 1991; Ingilæ *et al.*, 2000; Lund *et al.*, 2002a). Because little is known about the protective antigens in cod against *A. salmonicida,* further immunological investigation of the protective antigens and their impact on protective immunity is important for Atlantic cod vaccine development.

5.3 Side-effects[3]

In this study, the results of the side-effects of the different vaccine formulations showed variations depending on the adjuvant used. The freunds incomplete adjuvants (FIA) led to severe peritoneal adhesions and displayed the highest score of side-effects as the internal organs were firmly attached to the abdominal wall and surrounded by non-transparent membranes. Aluminium hydroxide (Alhydrogel) led to inflammation and adherence at the site of injection, but no adhesions were observed elsewhere in the body cavity. Also, the results showed no side-effects in unvaccinated fish or in fish received bacterin or CpG alone or in combination. These results are in consistence with previous reports and are well known for freunds adjuvants, which leads to visible side-effects in vaccinated fish (Midtlyng *et al.*, 1996a; Midtlyng, 1997b; Poppe and Breck, 1997).

The score used in this study is described in relation to extend of adhesions of the inner organs and the highest score indicated by (2) followed by less degree score (1) and score (0) indicate no side-effects. This score is not in accordance with the Speilberg score used in prevoius studies of salmon (Midtlyng *et al.* 1996a) because side-effects in cod appear with the same degree of severity in all investigated fish. These findings confirm that the intra-abdominal adhesions of the internal organ and the abdominal wall may affect the commercial quality of farmed cod and in particular the further used of the cod liver.

[3] Side-effects is a term used for various adverse or undesirable effects arises from medical treatments including vaccination (Midtlyng, 1998).

In spite of the presence of severe intraperitoneal lesions in fish receiving oil adjuvant no reduction in the weight was observed in these groups. However, vaccines including aluminium hydroxide yield less protection level, and seem to have bad influence in the weight of the cod. Also, this result is similar to the effect of Aluminium salts adjuvants in salmonids species (Horne *et al*., 1984; Midtlyng and Lillehaug, 1998b). In the present study, no side-effects were observed in fish received CpG with or without antigen. This finding is inconsistence with the previous report, which state that CpG has less toxic effect than other adjuvants (Weeratna *et al*., 2000).

The absence of the intra- abdominal lesions and the high level of protection in fish vaccinated with the antigen alone, support that the use of oil-based vaccines should be discontinued as a vaccine component (Classen *et al*., 1992; Midtlyng, 1997). There is a growing awareness that the vaccine side-effects has a negative effect on fish health and production. The reduction and elimination of side-effects with maintenance of long- term efficacy is never the less a decent goal, and it seems to be a possible goal for the vaccines development against atypical furunculosis in farmed Atlantic cod.

6. CONCLUSIONS

The aim of this study was to examine several adjuvants, alone or in combination, for their ability to enhance protective immunity against atypical furunculosis and to evaluate the degree of side-effects that they cause in Atlantic cod.

The results obtained in cod demonstrated that intraperitoneal vaccination using adjuvanted bacterins is effective against atypical furunculosis in cod, and that the use of atypical *A.salmonicida* vaccine without adjuvant as well as FIA-containing vaccines protect Atlantic cod effectively. The presence of visible injection-site lesions was associated with vaccines containing FIA and Aluminium hydroxide alone. In contrast to previous studies in vibriosis, Atlantic cod produced specific antibodies against atypical *A.salmonicida*. The level of protection found correlated with the specific antibody responses in all the vaccinated groups where antigen was present.

In addition, the following conclusions were derived from the result obtained in this study:

- The intra-abdominal (i.p.) vaccination of Atlantic cod with the bacterin in saline gives a good protection without showing any symptoms of side-effects. This leads to conclude that it is effective to protect the Atlantic cod.
- Antigen and CpG DNA adjuvant, with or without oil protect Atlantic cod against atypical furuculosis. CpG DNA used alone gave a very high mortality. Therefore, it is difficult to give the conclusive effect of the CpG as an adjuvant in cod.
- The seriously side-effects of adjuvanted vaccines were found in Atlantic cod. Vaccination with FIA leads to adhesion between internal organs and the abdominal wall, inflammatory reaction due the presence of the rest of the vaccines in the form of spots. In addition, Aluminium hydroxide was found to suppress the growth and induced also inflammatory adhesion. These adjuvant systems should be avoided in the development of anti-atypical furunculosis vaccines and more effective alternative formulation is thus indispensable in cod. Further assessments of the side-effects after 6 months post vaccination are needed.
- Specific antibody responses were demonstrated in all groups vaccinated with atypical *A.salmonicida* bacterin. The responses were strongest in groups receiving bacterin in FIA.

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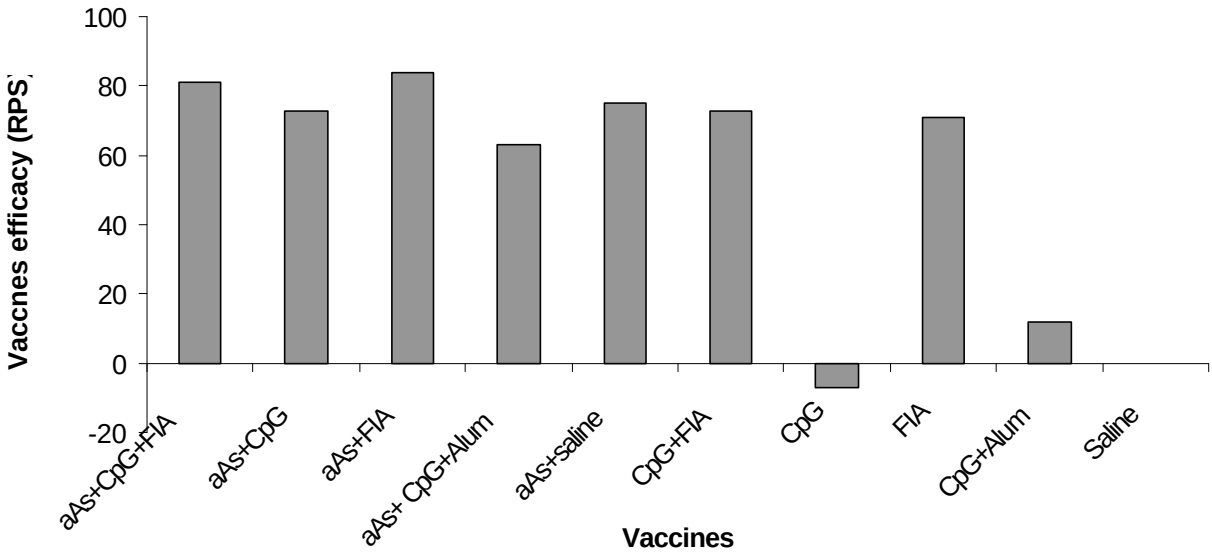
8. APPENDICES

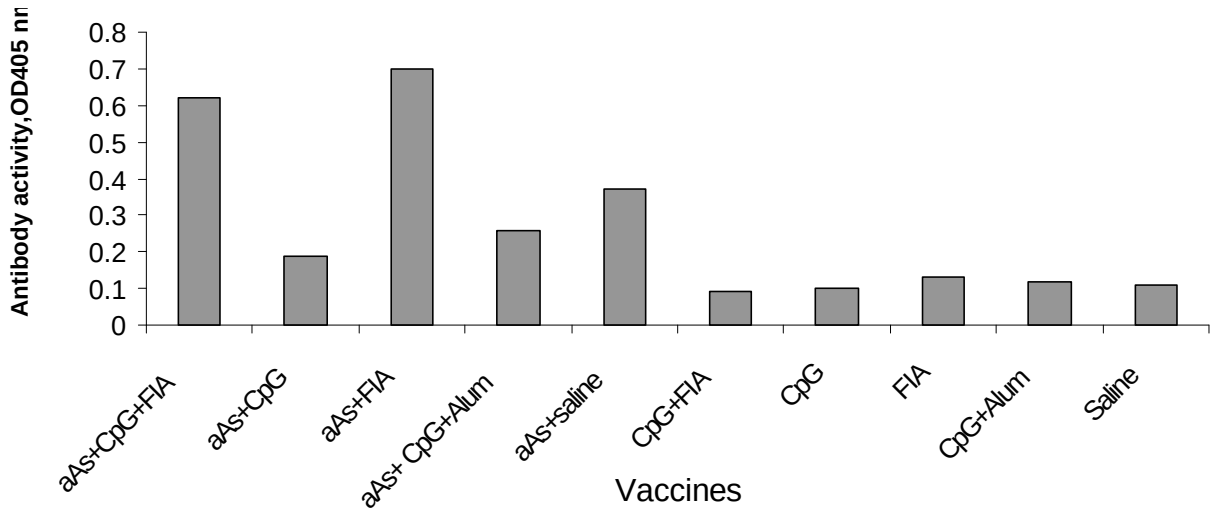
Appendix A:

Vaccines efficacy (RPS) in Atlantic cod (*Gadus morhua* L.) vaccinated with atypical *A. salmonicida* vaccines and challenged with atypical *A. salmonicida* aAs 4099, 12 weeks post vaccination.



Appendix B:

Antibody responses in Atlantic cod against atypical *A.salmonicida* measured in ELISA. The sera were diluted 1:1600.



Appendix C

Appendix C-1:

χ^2- test comparing the differences in mortality between the group 1-9 and the control group (saline). Degree of freedom of comparing two groups (df = 1).

Comparisons		χ^2	P- value
aAs+CpG+FIA (Gr1)	Saline (Gr10)	33.5695	P < 0.001
aAs+CpG (Gr2)	Saline (Gr10)	33.5695	P < 0.001
aAs+FIA (Gr3)	Saline (Gr10)	37.5282	P < 0.001
aAs+CpG+Alum (Gr4)	Saline (Gr10)	37.5282	P < 0.001
aAs+saline (Gr5)	Saline (Gr10)	27.5686	P < 0.001
CpG+FIA (Gr6)	Saline (Gr10)	26.0903	P < 0.001
CpG (Gr7)	Saline (Gr10)	0.2431	n.s.*
FIA (Gr8)	Saline (Gr10)	25.1537	P < 0.001
CpG+Alum (Gr9)	Saline (Gr10)	0.6403	n.s.*

* n.s = not significant

Appendix C-2:

χ^2 - test comparing differences in mortality between the groups received atypical *A.salmonicida* bacterin in combination either with CpG, FIA, CpG+ Alum (Alhydrogel) or CpG+FIA and the groups received the respective adjuvant alone. Degree of freedom of comparing two groups (df =1).

Comparisons		χ^2	P- value
aAs+CpG+FIA (Gr1)	CpG+FIA (Gr 6)	0.8035	n.s.*
aAs+CpG (Gr2)	CpG (Gr7)	40.1957	P < 0.001
aAs+FIA (Gr3)	FIA (Gr8)	1.9619	n.s*
aAs+CpG+Alum (Gr4)	CpG+Alum (Gr9)	13.5064	P < 0.001
aAs+saline (Gr5)	Saline (Gr10)	27.5686	P < 0.001

* n.s.= not significant

Appendix C-3:

χ^2- test comparing differences in mortality between the group1-5 with each other.

Degree of freedom of comparing two groups (df =1).

Comparisons		χ^2	P- value
aAs+CpG+FIA (Gr1)	aAs+CpG (Gr2)	0.8035	P < 0.1
aAs+CpG+FIA (Gr1)	aAs+FIA (Gr3)	0.1188	P < 0.1
aAs+CpG+FIA (Gr1)	aAs+CpG+Alum (Gr4)	2.8998	P < 0.1
aAs+CpG+FIA (Gr1)	aAs + saline (Gr5)	0.4953	P < 0.1
aAs+CpG (Gr2)	aAs+FIA (Gr3)	0.1188	P < 0.1
aAs+CpG (Gr2)	aAs+CpG+Alum (Gr4)	2.8998	P < 0.1
aAs+CpG (Gr2)	aAs + saline (Gr5)	0.4953	P < 0.1
aAs+FIA (Gr3)	aAs+CpG+Alum (Gr4)	4.2103	P < 0.05*
aAs+FIA (Gr3)	aAs + saline (Gr5)	1.1081	P < 0.1
aAs+CpG+Alum (Gr4)	aAs + saline (Gr5)	1.0358	P < 0.1

* P-value < 0.05, mean there is significant difference between group 3 and group 4.

Appendix D:

The mean weight of the fish at time of vaccination and 12 weeks post vaccination. (Mean weight ± S.D.)

Group No.	Fish mean weight (g) before and after vaccination	
	At time of vaccination	12 weeks post vaccination
Gr 1	33.9± 9.3	59 ± 16.5
Gr 2	38.9 ± 10.7	74.5 ± 24.5
Gr 3	32.3 ± 6.5	78.3 ± 26.7
Gr 4	35.3 ± 8.8	44.2 ± 13.6
Gr 5	35.7 ±6.6	73.9 ± 27.6
Gr 6	35.3 ± 8.0	66.3 ± 13.5
Gr 7	35.3 ± 9.9	79.8 ± 25.5
Gr 8	31.4 ± 5.3	65.0 ± 24.2
Gr 9	31.8 ± 11.4	59.9 ± 16.0
Gr 10	34.0 ± 9.3	67.7 ± 16.3

Appendix E:

The fluorescent tagging system (Northwest marine technology, inc.) for the 10 groups of Atlantic cod vaccinated against atypical *A. salmonicida* used to tag the fish before the challenge experiment.

Group	**Colour**	**Direction**	**Location**
1.	Green	Left	Operculum
2.	Orange	Left	Operculum
3.	Yellow	Left	Operculum
4.	Red	Left	Operculum
5.	Yellow/Yellow	Left/Right	Operculum
6.	Green	Right	Operculum
7.	Orange	Right	Operculum
8.	Yellow	Right	Operculum
9.	Red	Right	Operculum
10.	Red/Red	Left/Right	Operculum

Appendix F:

The temperature during the vaccination and challenge experiment. The challenge was performed at week 12 post vaccination.