Adaptive Humoral Immune Response in Lumpfish, *Cyclopterus lumpus* L.

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LIST OF ACRONYMS

Abbreviation	Full name
Ab	Antibody
Ag	Antigen
AMM3	Alpha Marine Micro 3
APCs	Antigen Presenting Cells
APS	Ammonium Persulfate
AsaP1	*A. salmonicida* subsp*. achromogenes protease* 1
BCR	B-Cell Receptor
BSA	Bovine Serum Albumin
C	Constant
C1	Initial concentration
C2	Final concentration
CD40L	Cluster of differentiation 40 Ligand
CDRs	Complimentary Determining Regions
cm	Centimeter
Co.	Cohabitation
DNA	Deoxyribonucleic acid
ECP	Extracellular product
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
Eq.	Equation
Fig.	Figure
g	Gram
H	Heavy
HiB	Høyteknologisenteret i Bergen
HMW	High Molecular Weight
HPR	Horseradish Peroxidase
Hz	Hertz

Abbreviation	Full name
i.p.	Intraperitoneal
Id. No.	Identity number
Ig	Immunoglobulin
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgT	Immunoglobulind T
IL	Interleukin
ILAB	Industrilaboratoriet
kDa	Kilo Dalton
L	Light
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
M	molarity
m^3	Meter Cube
mg	Milligram
MHC	Major Histocompatibility Complex
ml	Milliliter
mm	Millimeter
MOMP	Major Outer Membrane Protein
MW	Molecular Weight
N	Number
ng	Nano gram
nm	Nanometer
ns	Not significant
OD	Optical density
ODs	Optical densities
OM	Outer membrane
OPD	o-phenylenediamine
PAMPs	Pathogen Associated Molecular Patterns

Abbreviation	**Full name**
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline – Tween
PRR	Pathogen Recognition Receptor
Rf	Relative distance
RNA	Ribonucleic acid
rpm	Revolution Per Minute
RPS	Relative Percent of Survival
RT	Room Temperature
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
spp.	Species
SRID	Single Radial Immunodiffusion Assay
Subsp.	Subspecies
TBS	Tris – Buffered – Saline
TCBS	Thiosulfate – citrate – bile salts sucrose
TD	Thymus dependent
TEMED	Tetramethylethylenediamine
TGS	Tris-Glycine-Sodium-dodecyl-sulfate
T_H	T Helper cells
TI	Thymus independent
TLR	Toll – like Receptors
TSB	Tryptic Soy Broth
TTBS	Tween Tris – Buffered – Saline
V	Variable
V1	Initial volume
V2	Final volume
VIE	Visible Implant Elastomer
WB	Whole Bacteria
µ	Micro

ABSTRACT

The lumpfish, *Cyclopterus lumpus* L. is an emerging and most promising aquaculture fish currently used for a biological control against sea lice in salmonids. However, the lumpfish is prone to a number of bacterial diseases including vibriosis, atypical furunculosis and pasteurellosis. The use of vaccines in aquaculture has been one of the best prophylactic measures against fish bacterial diseases; vaccines can protect the lumpfish from bacterial diseases. Thus, it is essential to investigate the adaptive humoral immune response of the lumpfish in order to develop protective vaccines against the bacterial diseases.

This work is the first to study the adaptive humoral immune response of the lumpfish in response to immunization with formalin inactivated whole bacteria (WB) antigens and challenge with different Gram-negative bacteria after vaccination or without vaccination. Immunization was conducted against two atypical *A. salmonicida, V. anguillarum* O2, *Pasteurella* spp. and *V. ordalii.* Analysis of the different lumpfish antisera using sonicated WB antigens by ELISA and western blot revealed the presence of strong specific or cross-reactive antibodies two weeks post immunization. The antisera to both atypical *A. salmonicida* strains had antibodies that detected common antigenic components directed to ca. 10, 13, 20, 22, 28, 50 and 55 kDa proteins. However, antibodies to one of the atypical strains (8546) did not recognize the 50-kDa protein from the heterogeneous antigen (AL20460) on the western blot. The absence of the 50-kDa protein in one of the atypical *A. salmonicida* may contribute to differences in the level of specific antibody response.

Antibodies to strain AL122 (*V. anguillarum* O2) reacted strongly with a 14-kDa antigenic component of heterogeneous antigens from strains 8752 (*V. anguillarum* O1) and strain 8657 (*V. ordalii*). The *Pasteurella* spp. induced the highest level of antibodies directed to 100 and 20 kDa proteins of a homogeneous antigen. Moreover, antibodies to *Pasteurella* spp. reacted with 100, 73, 37 and 20 kDa protein components of a heterogeneous antigen from *P. skyensis.* However, *V. ordalii* induced the lowest antibody response; no specific antibodies were detected in antisera to *V. ordalii* by the western blot analysis.

The lumpfish's specific humoral immune system showed strong antibody response to vaccination post challenge (i.p.) with strains 8546 (atypical *A. salmonicida*), AL20460 (atypical *A. salmonicida*), 8752 (*V. anguillarum*) and *Pasteurella* spp. The trivalent and the commercial AMM3 vaccines against atypical furunculosis showed higher antibody levels after challenge with strain 8546 (atypical *A. salmonicida*) than challenge with strain AL20460 (atypical *A. salmonicida*). The monovalent vaccines had similar effect on antibody levels after challenging with either of the atypical *A. salmonicida* strains. The trivalent and monovalent vaccines against *Vibriosis* induced similar amount of antibodies post challenge with strain 8752 (*V. anguillarum*). However, the non-vaccinated groups, in general, displayed low antibody levels post challenge with post challenge with strains of the atypical *A. salmonicida*, 8752 (*V. anguillarum* O1), and *Pasteurella* spp. by any of the challenge exposures (bath, i.p., or cohabitation). These results indicate that the use of the vaccines in the lumpfish may play a protective role against some of the bacterial diseases; however, further studies are needed.

Moreover, total serum IgM in the lumpfish was quantified by single radial immunodiffusion assay. The mean total IgM (mg/ml) post immunization for the different groups was estimated at the range of 1.02 to 0.41 mg/ml, which is in the range also found in other fish species.

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1. INTRODUCTION

1.1 The lumpfish, Cyclopterus lumpus L.

The lumpsucker or lumpfish, *Cyclopterus lumpus* L. (Linnaeus, 1758) is one of the 28 fish species identified under the family of *Cyclopteridae* (Mecklenburg and Sheiko, 2003). It is the sole species in genus *Cyclopterus* (Davenport, 1985). They are marine, mostly benthos inhabiting continental shelves and upper slopes. Females lay their eggs in shallow coastal waters or may migrate hundreds of miles; the males protect the eggs after spawning. Benthic organisms such as polychaetes, crustaceans and molluscs are major food sources for the lumpfish, while some feed on slow moving pelagic organisms such as medusae and ctenophores (Mecklenburg and Sheiko, 2003). The *Cyclopterus lumpus* L. has an extensive range of distribution along the coasts of the Arctic Ocean, northern sections of the North Pacific and North Atlantic oceans (Davenport, 1985, Mecklenburg and Sheiko, 2003, Mecklenburg et al., 2011). Fig. 1 (Davenport, 1985) shows a map of the lumpfish distribution in the northern part of the Atlantic Ocean. In many western North Atlantic countries, *Cyclopterus lumpus* L. is an important commercial fish, in which female lumpfish are caught for caviar production (Mecklenburg and Sheiko, 2003). Recently, the lumpfish has become an emerging and most promising cleaner fish to be used for biological control against sea lice, *Lepeophteirus salmonis* Krøyer, in salmonid aquaculture.

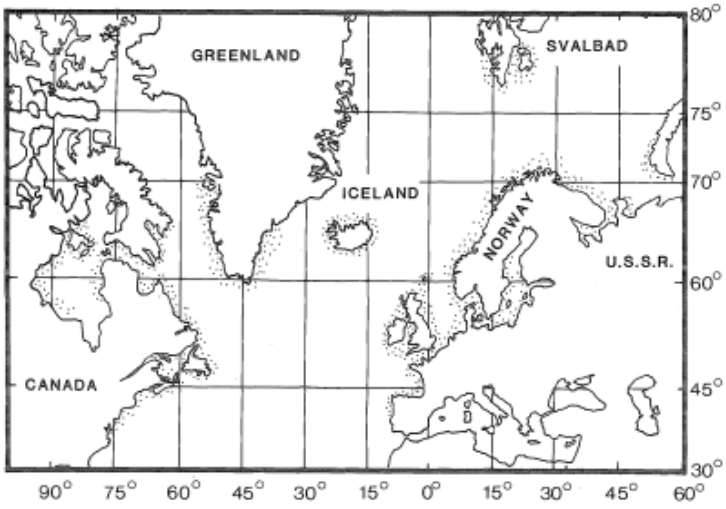


Fig. 1. A map of the lumpfish, *Cyclopterus lumpus* L., distribution. The small black dots indicate for the lumpfish's breeding ground (Davenport, 1985).

The salmon louse is a cause of serious environmental, welfare and economic problems that may compromise the sustainability of the salmon aquaculture industry (Jimenez et al., 2012, Denholm et al., 2002). For many years, the industry has relied on topical and oral administration of chemotherapeutants for the control of salmon lice (Torrissen et al., 2013). However, increased use of chemicals has led to the development of resistant lice (Jimenez et al., 2012). Thus, such concerns coupled with public preference to high quality fish product and immediate necessity for effective and sustainable sea lice control have prompted the industry to use environmentally friendly cleaner fish as biological control against sea lice infestation (Treasurer, 2002). At present, the principal cleaner fish in use for biological sea lice control include Ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris* L.) and the lumpfish (*Cyclopterus lumpus* L.). The wrasses are not without limitations; they are incapable of tolerating low temperature less than 6 ºC making them unsuitable candidates in cold regions (Sayer and Reader, 1996), like in Northern Norway. The lumpfish, however, can tolerate low water temperature (Imsland et al., 2014). According to the 2013 report on statistics of fish farming by the Norwegian Department of Fisheries (Fiskeridirektoratet), the farming of lumpfish in Norway has been growing since 2011; the lumpfish constituted 95% of all the cleaner fish produced in 2013.

In the same year, 1.95 million farmed lumpfish were sold for delousing in sea cages of Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* W.) (Fiskeridirektoratet, 2014).

1.2 Bacterial diseases in lumpfish

However, like any other animal husbandry, the lumpfish are also prone to a number of bacterial pathogens. It has been reported that several bacterial pathogens have been isolated and identified in both wild and captive lumpfish, including atypical *Aeromonas salmonicida* (*A. salmonicida*), *Pseudomonas anguilliseptica* (*P. anguilliseptica*), *Pasteurella* species (*Pasteurella* spp.) and diverse species of *vibrio* bacteria such as *Vibrio anguillarum* (*V. anguillarum*), *Vibrio ordalii* (*V. ordalii*), *Vibrio splendidus* (*V. splendidus*), *Vibrio tapetis* (*V. tapetis*), *Vibrio wodanis* (*V. wodanis*) and *Vibrio logeli* (*V. logeli*) (Nilsen et al., 2014). According to Nilsen et al., (2014), 310,043 (33%) mortalities were registered out of the total 934,935 cleaner fish stocked in 17 marine sites within a period of six months in 2013. The most serious mortality episode after transfer to sea cages was related to acute outbreak of bacterial infections by atypical *A. salmonicida*, *V.* anguillarum or *Pasteurella* spp. The rate of mortality (32 – 48%) was highest for the lumpfish (Nilsen et al., 2014).

Atypical *frunculosis: A. salmonicida* is one of the destructive bacterial fish pathogens responsible for great economic losses in several fish farms in the world (Diamanka et al., 2014). It is a facultative, gram negative, anaerobic, immobile, fermentative and rod-shaped bacterium (Gudmundsdottir, 1998). According to Bergey's manual of systematic bacteriology, there are five subspecies (subsp.) of *A. salmonicida,* which include subsp. s*almonicida,* subsp. *achromogenes,* subsp. *masoucida,* subsp. *smithia* and subsp. *pectinolytica* (Martin-Carnahan and Joseph, 2005). The *A. salmonicida* spp. *salmonicida* is the aetiological agent of typical frunculosis in salmonids and mentioned as typical, whereas the other subsp. including many other strains that do not fall into the taxonomic category of *A. salmonicida* spp. salmonicida are described as atypical *A.*

salmonicida (Gudmundsdottir, 1998, Wiklund and Dalsgaard, 1998). Unlike the typical *A. salmonicida spp. salmonicida,* the atypical *A. salmonicida* strains are heterogeneous showing wide variations with respect to their biochemical, molecular and virulence features (Austin et al., 1998). The atypical *A. salmonicida* are responsible for profound and extensive ulcers and systemic infections in a wide range of farmed and wild fish species in both freshwater and marine ecosystems (Gudmundsdottir, 1998, Wiklund and Dalsgaard, 1998). Different strains of the atypical *A. salmonicida* have been identified in more than 20 farmed and 30 wild fish species (Wiklund and Dalsgaard, 1998). These bacteria affect particularly fish in the Northern hemisphere such as in Canada, USA, Japan, and central and northern Europe including the Scandinavian countries (Wiklund and Dalsgaard, 1998). The atypical strains have also been isolated from Norwegian farmed and wild fish species with the subsp. *achromogenes* being the most abundantly scattered in Norway and perhaps globally (Sorum et al., 2000). They are regarded as the most significant pathogens of cleaner fish, including lumpfish, causing chronic diseases with granulomas in internal organs and ulcerative lesions (Johansen, 2013). Like many gram-negative bacteria, the virulence factor of atypical *A. salmonicida* is possibly associated with various cellular components found in its membrane, which is composed of a peptidoglycan and an outer membrane (OM) (Gudmundsdottir, 1998). At the surface of the OM is a protein layer known as A–layer, which is believed to be a virulent factor. Strains without the A-layer display reduced virulence (Noonan and Trust, 1995). The A-layer plays an important role in defending the pathogen from phagocytosis (Trust et al., 1996), proteolytic actions (Chu et al., 1991), serum effects and accelerates the binding of immunoglobulins (Ig), porphyrins and a number of extracellular proteins (Trust et al., 1996). The Lipopolysaccharide (LPS), which molecularly and physically shields the cell, is made up of lipid A, a central oligosaccharide and the O-antigen or O-chain polysaccharide subunits. Unlike the typical strain, the O-chain polysaccharide subunits in the atypical strain is incomplete with either absence or presence of an O-acetyl group (Wang et al., 2007). The O-antigen is located at the outermost surface; hence, it is a region where specific interaction occurs with antibodies (Gudmundsdottir, 1998). Moreover, metallo-caseinase (AsaP1), a 20-kilodalton (kDa) exotoxin isolated from extracellular product (ECP) of some atypical strains including ssp. *achromogenes* is

deadly to fish. AsaP1 may induce protection against the atypical furunculosis (Gudmundsdottir and Magnadottir, 1997).

Vibriosis: The lumpfish are also susceptible to a group of bacteria belonging to genus *Vibrio*, which cause a disease condition known as *vibriosis* (Woo et al., 2011). The vibrio species are found everywhere in the aquatic environment (Haenen et al., 2014). Within the family of *Vibrionaceae* the vibrio that result in devastating economic loss in mariculture are *V. anguillarum*, *Vibrio salmonicida* (*V. salmonicida*), *V. ordalii* and *V. vulnificus.* The disease associated with *V. anguillarum* infection is referred to as classical vibriosisis, which is characterized by a typical haemorrhagic septicaemia in a number of economically important cold- and warm-water species of fish. Fish with classical vibriosis display symptoms of extensive septicaemia with bleeding on fins, protrusion of the eyes, corneal opacity, anorexia with discolored gills, which is a reflection of serious anaemia, and oedematous injuries in the hypodermis (Toranzo et al., 2005). Chemical, physical and biological stressors can trigger epidemics of *vibriosis* (Frans et al., 2011). *V. anguillarum* is a gram-negative, curved rod shaped (0.5x1.5 µm), motile with the help of a single polar flagellum, non-spore-forming, halophilic and facultative anaerobic bacterium, which grows in a medium containing 1.5 – 2% NaCl at a temperature of 20 – 30 ºC (Frans et al., 2011, Austin and Austin, 2007).

The virulence factors in *V. anguillarum* are perhaps linked to ECP exotoxins with proteolytic or haemolytic activities, LPS (O-antigen), siderophore and flagellum (Frans et al., 2011). The ECP exotoxins such as hemolysins can breakdown the host's erythrocyte membranes and hence, discharge of iron-binding proteins such as hemoglobin, transferrin and lactoferrin (Zhang and Austin, 2005). Another ECP exotoxin produced by the bacterium is a 36-kDa zinc metalloprotease (EmPA). EmpA is an essential virulence factor and involves in penetration and degradation of the mucus membrane (Norqvist et al., 1990, Denkin and Nelson, 2004). Afterwards, the infection becomes systemic, damaging vital organs such as the kidneys and liver (Frans et al., 2011). *V. anguillaurm* has a single flagellum made up of five subunits, in which one of the five subunits known

as flagellin A (flaA) is a virulent factor required for traversing the fish integument following invasion (Milton et al., 1996). The immune system of fish utilizes iron binding proteins such as transferrin, lactoferrin and ferritin making iron - a limiting growth factor - inaccessible for pathogens (Frans et al., 2011). However, *V. anguillarum* utilizes iron in iron-restricted environment by producing plasmid-mediated high-affinity iron chelating compounds known as siderophores. Genes contained inside plasmid pMJ1 of *V. anguillarum* encode proteins that facilitate biosynthesis of siderophore anguibactin and uptake of ferri-siderephore into the cytosol (Stork et al., 2002).

V. anguillarum has been considered as a very heterogeneous species (Kühn et al., 1996) comprising 23 O serotypes (O1 – O23) (Pedersen et al., 1999), however, only serotypes O1 or O2 and to a smaller degree serotype O3 have been related to vibriosis (Pedersen et al., 1999, Sørensen and Larsen, 1986). Serotypes O1 and O2 have worldwide distribution causing serious economic damage to several aquaculture farms, whereas, the other serotypes are considered as environmental isolates from sediments and water (Pedersen et al., 1999, Toranzo et al., 2005). Unlike serotype O1, which displays antigenic similarity, serotypes O2 and O3 show antigenic heterogeneities, each having two subgroups called O2a and O2b, and O3A and O3B, respectively. The serotype O3A is isolated from diseased fish, mostly from eel and ayu, while, O3B consists of environmental variants. The subgroup O2a can be detected from both salmonids and other fish species; whereas, subgroup O2b has been isolated from marine fishes only (Toranzo et al., 2005). According to the 2013 report regarding the status of fish health in Norwegian aquaculture, serotypes O1 and O2b had been spotted from diseased lumpfish in 2012 (Johansen, 2013).

V. ordalii is another causative agent of fish *vibriosis*. Initially, the bacterium was categorized as a strain belonging to *V. anguillarum* biotype 2 (Schiewe and Crosa, 1981). Like *V. anguillarum, V. ordalii* is also gram-negative, fermentative, curved rod-shaped (2.5-3.0x1.0 µm) and motile by a single polar flagellum. *V. ordalii* can be cultured in seawater agar and Thiosulfate-citrate-bile salts sucrose (TCBS) agar at 15 – 25 °C giving

creamy and circular (1-2mm) colonies after 4 – 6 days of incubation (Austin and Austin, 2007). Contrary to *V. anguillarum, V. ordalii* show antigenic homogeneity (Toranzo et al., 2005) based on plasmid profiling, ribotyping and serogrouping (Austin and Austin, 2007). Moreover, the vibriosis caused by *V. ordalii* is also characterized as haemorrhagic septicaemia, but with some pathological differences with the *vibriosis* caused by *V. anguillarum* (Austin and Austin, 2007, Toranzo et al., 2005). In the case of infections by *V. ordalii*, the bacteraemia grows slower than infections with *V. anguillarum.* Primarily, the bacterium was considered as causative agent of *vibriosis* in wild and farmed fish from USA, Japan and Australia (Woo et al., 2011); it has also been isolated from farmed Atlantic salmon in Chile (Toranzo et al., 2005). According to the Norwegian fish health report, *V. ordalii* has been diagnosed in many of the Norwegian lumpfish farms in 2013 (Johansen, 2013).

Pasteurellosis: Although diseases caused by the previously discussed bacteria have been well recognized for several years, some bacteria that belong to genus *Pasteurella* - a member of a family *Pasteurellaceae* - have emerged recently and have been affecting the fish farming industry (Birkbeck et al., 2002, Toranzo et al., 2005). *Pasteurella skyensis* (*P. skyensis*) was isolated from kidneys of farmed Atlantic salmon following disease outbreaks in Scotland and suggested as a causative agent of *Pasteurellosis* (Birkbeck et al., 2002). Affected fish displayed substantial cataracts, loss of weight, empty stomach and distinct white focal lesions in the kidney, spleen and heart. Besides, examination of peritoneal organs and swim bladder revealed the presence of pericarditis and peritonitis with granuloma and false membranes (Jones and Cox, 1999). Based on 16S rRNA and rpoB gene partial sequencing, two different serotypes (serotype 1 and 2) of *P. skyensis* have been isolated. Recently, several isolates with 97% resemblance in the 16SrRNA to the Scotish *P. skyensis* serotype 1 (strain 95A1^{T}) have also been detected in Norway (Reid and Birkbeck, 2014). The Norwegian isolate causes unusual disease in salmon known as ‘varracalbmi’ (Lapp for blood-eye). In the years 1989 – 2012, the bacterium had been intermittently found over a wide area extending from Hordaland in the south to Troms in the north (Johansen, 2014). In 2013, lumpfish in 16 separate

aquaculture facilities were diagnosed with systemic infections caused by a *Pasteurella* – spp. (Alarcón et al., 2014). Subclinical infections coupled with stress related to vaccination, transport or transfer to a new environment might exacerbate the rate of lumpfish mortality, and the mortality may reach 100% (Johansen, 2014). The isolates from the lumpfish have close phenotypic relations with the unspeciated *Pasteurella* spp., however, phenotypically different from the *P. skyensis* isolated from the diseased Atlantic salmon in Scotland. The placement of the Norwegian isolates as new species or subspecies within the family *Pasteurellaceae* has still been unknown (Alarcón et al., 2014).

1.3 Vaccination in fish

We have seen that the fish farming industry has suffered ravaging economic losses due to the different bacterial diseases. Consequently, the industry must take some measures to control the disease or prevent disease introduction into the facilities so that the industry would enjoy economic sustainability. One measure of disease control commonly employed by fish farmers would be to rely on using antibacterial drugs. With intensification of the Norwegian salmonid aquaculture in around 1980's, bacterial diseases, particularly vibriosis, cold-water vibriosis and furunculosis, were major problems. Thus, at the time where fish vaccines were not available, a maximum of approximately 50 tons of antibiotic drugs had been used in 1987 (Håstein et al., 2005) (fig. 2, (Harvest, 2015)). Meanwhile, the total fish production was only ca. 56,000 tones. However, following the introduction of effective vaccines in 1994 coupled with good aquaculture practices, the amount of drugs used dropped significantly to 1.4 tones, while at the same time fish production increased by almost three folds (Harvest, 2015). Now, annual fish production has continued to increase and 1.25 million tons of fish were produced in 2012, which was a 96% increase since 1987. Simultaneously, antibiotic use has decreased by 99.8% per ton of trout and salmon produced (Pharmaq, 2015). Therefore, the Norwegian success story clearly demonstrates that vaccination is an economically, ecologically and ethically preventive measure against fish diseases (Tafalla et al., 2013).

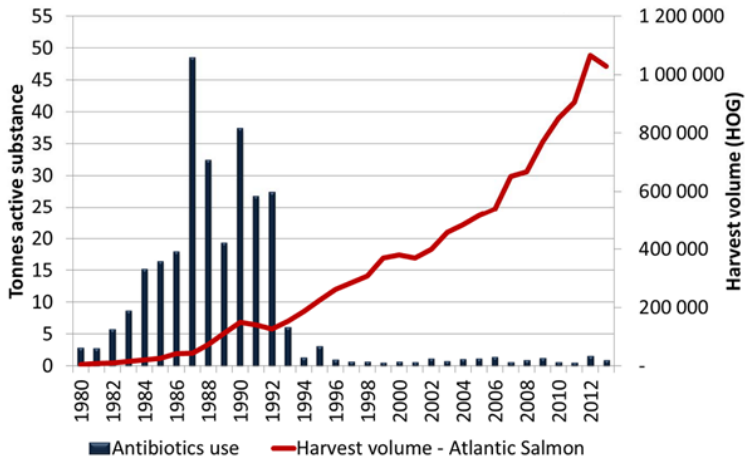


Fig. 2. Amount of antibacterial drugs used in Norwegian aquaculture due to outbreak of bacterial diseases in the 1980s and fish production from 1980 to 2012. Despite low levels of antibiotic use, the industry has enjoyed tremendous production increment since 1994 thanks to vaccination and some good husbandry practices (Harvest, 2015).

Nowadays, more than 17 species of fish in more than 40 countries are protected against 22 bacterial diseases and 6 viral diseases (Brudeseth et al., 2013). Most of the bacterial vaccines contain inactivated products (antigens) of the disease causing agents in oil adjuvants (Håstein et al., 2005). Vaccines can be delivered to the fish in three ways, namely injection mainly via intraperitoneal injection (i.p.), immersion by dipping or bath and oral administration (Gudding et al., 1999, Håstein et al., 2005). Each method has its own advantage or disadvantage with regard to the degree of protection, adverse side effects, practicality and cost (Gudding et al., 1999). In general, injectable vaccines provide the best protection against fish diseases (Håstein et al., 2005). According to PHARMAQ AS, around 2 billion salmonids in Norway have so far received oil based injectable vaccines containing formalin-inactivated antigens. To understand how vaccination or immunization protects the fish against the bacterial fish pathogens, it is

necessary to explore the fish immune system with particular focus on the stimulation of the specific humoral immunity (antibody production).

1.4 The immune system of fish

Most of the scientific knowledge concerning the fish immune system is limited to teleost fish; since the lumpfish is a new emerging species, there is very limited scientific review or research paper done with respect to their immune system. Therefore, this introductory review, as a whole, will discuss the immune system of fish based on information obtained from teleost fish and mammalian immunity. The fish immune system is physiologically comparable to that of advanced vertebrates, despite some dissimilarities. Besides, the fish immune system contains antigen receptors (immunoglobulins and T cell receptors), major histocompatibility complexes (MHC) and gene rearranging proteins (rag genes) (Fletcher and Secombes, 2010). However, fish lack lymphatic nodules and bone marrow (Zapata et al., 2006), which are the principal sources of blood cells in higher animals (Murphy, 2012); hence, other organs such as the kidney, spleen and thymus serve as major lymphoid organs. For instance, the head kidney, also called pronephros, in teleost fish is comparable to the bone marrow in mammals and is the chief spot for hematopoiesis (Zapata et al., 2006). Moreover, the head kidney is responsible for phagocytosis (Dannevig et al., 1994), antigen processing, antibody production (Brattgjerd and Evensen, 1996, Kaattari and Irwin, 1985) and immunological memory (Herraez and Zapata, 1986, Tsujii and Seno, 1990). The head kidney contains mostly B lymphocytes and macrophages combined in structures known as melanomacrophage centres (Press et al., 1994). The thymus is involved in production of T – lymphocytes that are engaged in allograft rejection, in prompting phagocytic activities by other cells of the immune system and antibody production by B lymphocytes (Bowden et al., 2005, Zapata and Amemiya, 2000). The main function of the spleen is to filter blood and process antigens (Espenes et al., 1995). Cells in the walls of the spleen participate in antigen phagocytosis by macrophage cells. Besides, antigens either in the form of antibody or metabolic products are stored in the spleen and play an essential role in immunological memory (Uribe et al., 2011). In general, the fish immune response, like in higher animals, is composed of

innate (non-specific) and adaptive immune (specific) responses (Magnadóttir, 2006); however, the adaptive immune response in fish is less advanced than that of higher animals (Warr, 1995). The primary function of the innate immune system is to protect the host against infection by controlling the disease causing microbes that have distinctive molecular structures on their cell walls or that trigger interferons and other general immune defenses (Murphy, 2012). The innate immune system in fish is composed of physical barriers (skin, epithelial tissue lines and mucus layers), cellular (macrophages, granulocytes, natural killer cells, etc.) and humoral elements (lysozyme, agglutinin, lectins, opsonins, antibacterial lytic enzymes, transferrin, etc.) (Magnadóttir, 2006, Swain and Nayak, 2009). The cells of the innate immune system have germline-encoded receptors known as pathogen recognition receptors (PRR) such as Toll like receptors (TLR), which are specialized to distinguish certain molecular structures, but not usually present in multicellular organisms. These molecular structures are collectively referred to as pathogen associated molecular patterns (PAMPs), which include polysaccharides, LPS, peptidoglycans and deoxyribonucleic acid (DNA) of bacteria or viral ribonucleic acid (RNA). In addition, the PRRs play a key role in the initiation of the adaptive immune system (Murphy, 2012).

Specific humoral immune response: The adaptive immune response takes place through interaction of intricate and highly specialized cells, proteins, genes and biochemical reactions that equip the host to communicate effectively with antibodies and effector cells in response to antigens with high affinity and specificity (Uribe et al., 2011). The adaptive immune system is mediated by T- and B lymphocytes (Fletcher and Secombes, 2010) and is comprised of cell - and antibody-mediated humoral responses. Antibody-mediated humoral responses occur when B cells are activated in response to antigen in cooperation with antigen presenting cells (APCs), specific T lymphocytes and various cytokines (fig. 3). Immunoglobulins (Ig) bound to the surface of the B cells act as B cell receptors (BCR) and have a wide range of specificities for different antigens derived from bacteria, or other pathogens. Antibody synthesis by the B cells can be instigated in two ways: either through thymus-independent antigens (TI) or thymus-dependent antigens

(TD). TI antigens are mostly products of microbes such as the bacterial LPS, which without the presence of T helper cells can directly induce B cells to generate specific antibodies, whereas TD antigens need the presence of T helper (T_H) cells to activate B – cells to make antibodies. Most proteins and peptides are TD antigens because they need activation from T_H cells in order to provoke an immune response (Weintraub, 2003, Möller, 2001). Professional APCs such as macrophages, dendritic cells, or B cells engulf and process antigens in their intracellular vesicles and present the peptide fragments on the surface in combination with the MHCII so that T_H cells would recognize them. The T_H cells produce interleukins (IL) that signal the B cells to undergo proliferation and clonal expansion to become antibody-producing plasma cells (Murphy, 2012). Here, collaboration between CD40 ligand (CD40L) or CD154 on the T cell and CD40 on the B cell is crucial for the TD responses (Fletcher and Secombes, 2010, Murphy, 2012). Before B cell activation, the same specific peptide fragment must trigger the CD4 T cells to make T_H cells. Each plasma cell or B-lymphocyte clone secretes a specific antibody in response to antigen having the same specificity (Murphy, 2012). TD antigen activated B and T lymphocytes to generate specific antibodies are considered memory cells because they provide long – lasting immune protection (Möller, 2001) and allow the immune system to react more quickly, vigorously and efficiently to the same pathogen during reinfection and protect the host from disease (Murphy, 2012).

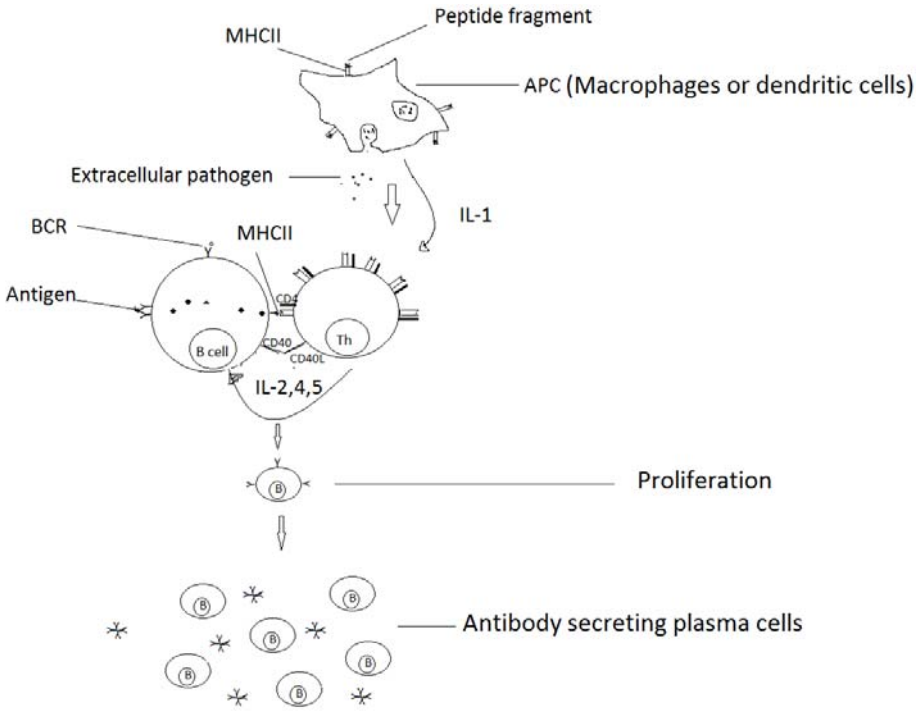


Fig. 3. TD cell activation of B cells. Professional APCs phagocytize extracellular pathogens (bacteria), process them inside intracellular vesicles and present the peptide fragments in combination with MHCII. The T lymphocyte recognizes the peptide-MHCII complex through its cell-surface CD4 receptor and divides into T_H effector cells. The BCR also recognize antigens, internalize and present them with MHCII; the T_H recognizes the peptide – MHCII complex and produces cytokines (IL-2,4,5) signaling B cells to undergo proliferation and clonal expansion (Murphy, 2012).

Structure of antibody, its specificity and antibody – antigen interaction: In understanding the specificity of antibodies to antigens, knowledge of the structure of an antibody molecule is crucial. A typical antibody molecule is similar to a letter Y, which is constructed of four polypeptide chains: two identical light (L) and two identical heavy (H) chains connected by disulfide bonds (hinge region) (fig. 4). The L and H chains can be divided into two distinctive regions: the variable (V) region found at the tips of each chain towards the amino acid terminal and the constant (C) region pointed in the direction of the carboxy-terminal. The V and C regions of an antibody molecule perform different

functions; the former is involved in recognition and binding of antibodies by its antigenic binding site (paratope), while the later has an effector function (Murphy, 2012, Lucas, 2003). As its name indicates, the amino acid sequences in the V – region differ significantly between different antibody molecules. The variability of the amino acid sequences is concentrated at the tip of the variable domains particularly in three amino acid segments known as hypervariable regions. Both the V_L and V_H domains constitute six hypervariable segments, in which altogether, form the entire antigen-binding site. These hypervariable regions are often termed as complementarity-determining regions (CDRs); various combinations of these regions decide the antigen specificity of the antibody. Uniform residue sequences outside the CDRs, which is called framework, do not make bonds with antigens, but control the three – dimensional folding of the Ig, provide the bending of the V region and support the structure of the binding-site. Moreover, the CDR structures can create different binding shapes in thier surface according to the structure of the antigens. The involvement of all binding sites of the antibody with the antigen and their combined strength is called avidity (Murphy, 2012, Kumagai and Tsumoto, 2001). On the other hand, some antibodies raised against certain antigens may often cross react with other closely related antigens or occasionally with unrelated antigens (Murphy, 2012). In some cases, greater affinity can be attained for antigens other than the original one (Kumagai and Tsumoto, 2001).

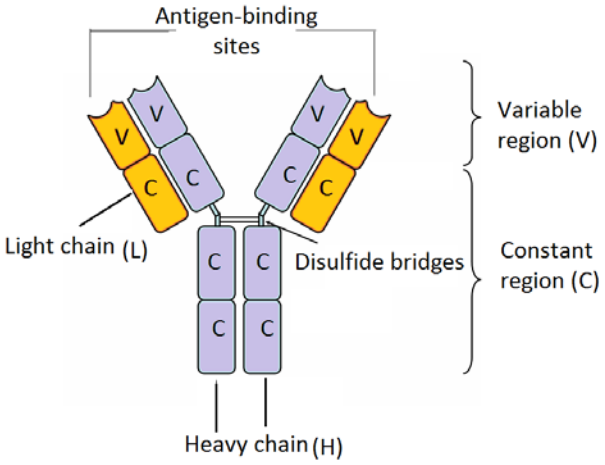


Fig. 4. Typical structure of an antibody molecule.

Although antibodies can virtually recognize any structure as antigen, some antigens fail to elicit immune response, i.e. they are not immunogenic. Proteins are good immunogens and can provoke entirely advanced adaptive immune responses because they have the capability to engage T cells. Some proteins can serve as carriers for carbohydrates, nucleic acids and other kinds of molecules (Murphy, 2012). During immunization, the immunogenicity of less immunogenic antigens can be enhanced by attaching the antigens with protein carriers in the presence of adjuvants (Ellis, 1988, Murphy, 2012). Moreover, larger, structurally more complex, aggregated and more distant protein antigens are required to provoke entirely advanced specific humoral responses. The extent of antibody response is also dependent on antigen dose. Low levels of antigens (below threshold level) cannot elicit immune response, but a gradual increase in antibody takes place with rising antigen dose until it reaches a plateau. The response starts to decline with increasing antigens above the threshold level; the immune response may cease following administration of very high antigen concentration (Murphy, 2012).

Fish IgM: So far, only three Ig classes, namely IgD (Wilson et al., 1997), IgT (Danilova et al., 2005, Hansen et al., 2005) and IgM (Acton et al., 1971) have been identified in fish. The IgM in teleost fish is produced by plasmablasts and plasma-like cells found in the anterior kidney and plays a pivotal role in stimulating memory IgM responses (Ye et al., 2011); it is the most dominant Ig in plasma (Warr, 1997). IgM shows significant structural and biochemical diversities among different species (Magnadóttir, 1998). For example, higher vertebrates and cartilaginous fish have pentameric IgM (Kunihiko et al., 1984), whereas it is hexameric in some amphibians (Hsu and Du Pasquier, 1984). The teleost IgM is mainly tetrameric consisting of eight antigenic binding sites (Acton et al., 1971). Some fish may contain monomeric IgM in their sera, but tetrameric IgM is more efficient than the monomeric one in triggering the complement system due to variations in the structure of their Fc portions (Elcombe et al., 1985). Unlike the mammalian IgM, the IgM of many fishes lacks the J – chain, a polypeptide responsible for IgM polymerization (Magnadóttir, 1998); however, there are some evidences for its presence in catfish

(Mestecky et al., 1975) and rainbow trout (Sanchez et al., 1989). The H and L chains of each monomer of the tetrameric Ig have a molecular weight of 60 – 77 and 23 – 26 kDa, respectively (Van Muiswinkel and Woo, 1995). The amount of serum IgM in fish may greatly vary from species to species (Castro et al., 2013). In addition, parameters like stress (Cuesta et al., 2004), size, age, water quality and seasonal variations may influence the level of Ig in fish (Scapigliati et al., 1999).

Immunization and immune response: The occurrence of specific antibody response can be monitored by examining sera of immunized, vaccinated or pathogen challenged fish. The magnitude of the specific antibodies increases with successive immunization of the animal with the same antigen. The initial response obtained during the first immunization is termed as primary antibody response. The primary antibody response is dominated by the production of IgM. The immune response can further develop into secondary or tertiary antibody responses with subsequent booster immunization at different times (fig. 5) (Ellis, 1988). A little amount of specific antibody is produced during first immunization at time zero. After some days, the antibody builds up until it reaches a peak, and then, plateaus and starts to decrease slowly. With the second immunization, very rapid and higher amount of antibodies having greater antigen binding affinity are generated. The amplified antigen binding affinity is known as affinity maturation (Murphy, 2012). Unlike higher vertebrates, affinity maturation does not exist in teleost fish; however, immunological memory with increased and improved secondary antibody responses with high specificity to antigens has been demonstrated (Fletcher and Secombes, 2010).

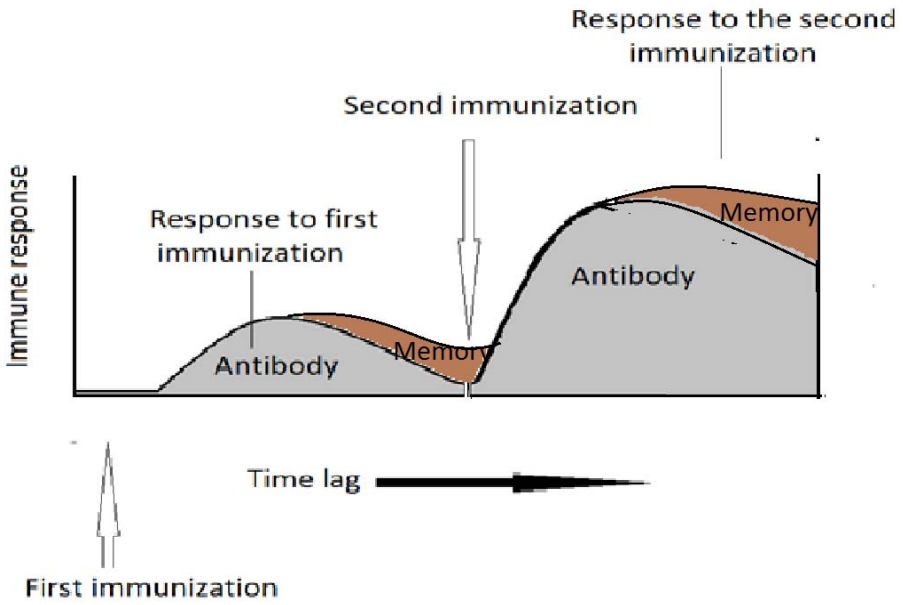


Fig. 5. Antibody responses during first and second immunization and the formation of memory cells (Ellis, 1988).

1.5 Objectives

Investigation of the specific humoral immune response in the lumpfish is of paramount importance in developing an environmentally and welfare friendly protective vaccines against diseases caused by bacterial pathogens. Yet, little is known about the specific humoral immune response of the lumpfish. Thus, the aim of this study is to:

- To investigate the presence of specific antibody response in sera of the lumpfish after immunization, vaccination and challenge with different bacterial pathogens.
- To test the occurrence of cross-reactive antibodies between different bacterial strains and
- To estimate total serum IgM in the lumpfish.

2. MATERIALS AND METHODS

All procedures and methods in this thesis are done according to a protocol used by the Fish immunology group at the University of Bergen. The Norwegian Animal Welfare Research Authorities approved the challenge experiment that was carried out in the lumpfish.

2.1 Fish and rearing conditions

Lumpfish, *Cyclopterus lumpus* L., (800 g; 21cm, (n=51)) were used in the immunization experiment. The fish were obtained from Fjordforsk AS in Sogndal and kept in a green 500 liter ($1m^3$) fiberglass tank in the rearing facilities of Industrilaboratoriet (ILAB) in Bergen at the High Technology Center (HiB) inside the University of Bergen. The fish were reared under normal optimal conditions at a temperature of 12 ºC, salinity of 34 ‰ and a photoperiod of 12:12 (light:dark). The fish were fed with a commercial dry feed 3mm pellet (BioMar) at 2% of their biomass using an automatic feeder. No sign of infection or mortality was observed during the experimental period.

2.2 Bacteria and antigens

Seven different Gram – negative bacterial pathogens listed in table 1 were used in this experiment. The bacteria were harvested from late exponential growth phase and grown in 50 ml tryptic soy broth (TSB) with or without additional NaCl depending on the growth requirement of the different bacteria. They were incubated at 21 ºC with continuous shaking at 200 rpm. Bacterial growth was checked and turbidity of the media was measured at 600 nm with a spectrophotometer (HITACHI U-1100). The growth for the atypical *A. salmonicida* strains was measured at 340 nm after resuspension in NaOH due to the agglutinating nature of this bacterium (Aakre et al., 1994). The bacterial suspension was washed three times in Phosphate Buffered Saline (PBS) by centrifugation at 2500 x g for 15 minutes following the decanting of the supernatant and

addition of PBS. The cells were re-suspended in a drop of distilled water and frozen at -20 ºC.

The frozen whole bacterial (WB) cells were freeze – dried; a stock solution of 1 mg/ml antigen was prepared by dissolving 2 mg of the freeze-dried bacteria in 2 ml PBS with 5M Ethylenediaminetetraacetic acid (EDTA) (pH 7.3). The bacterial solution was sonicated three times for 20 seconds each at 40 - 60 Hz by ultrasonic homogenizer (Teknisk Kjemisk). The stock solution was divided into aliquots and stored at -20 ºC.

Table 1. List of Gram negative fish bacterial pathogens used for immunization, challenge and antigens in ELISA and western blot and for SDS – PAGE profiles.

Id. No.	**Bacteria**	**Source**
8546	Atypical *A. salmonicida*	Lumpfish
AL20460	Atypical *A. salmonicida*	Lumpfish
AL122	*Vibrio* (*Listonella*) *anguillarum* O2	Cod
8752	*Vibrio* (*Listonella*) *anguillarum* O1	Lumpfish
8657	*V. ordalii*	Lumpfish
-	*Pasteurella* spp.	Lumpfish
-	*P. skyensis*	Salmon

2.3 Immunization

The fish were starved for 24 hours prior to immunization. For identification of vaccine groups the fish were marked with Visible Implant Elastomer, VIE, silicone implants (North West Marine Technology, Inc.) on the forehead. Four fluorescent dyes - orange, red, blue and green - were used.

Immunization was carried out against formalin inactivated WB from strains 8546 (atypical *A. salmonicida*), AL20460 (atypical *A. salmonicida*), AL122 (*V. anguillarum* O2), 8657 *Pasteurella* spp. and 8657 (*V. ordalii*). The bacterial strains had been isolated after outbreak of disease in the lumpfish, except for AL112 (*V. anguillarum* O2), which was isolated from Atlantic cod. The fish were manually immunized three times with 100 µl of the antigen by intraperitoneal (i.p.) injections. The first immunization contained formalin inactivated bacteria emulsified in a mineral oil adjuvant (Freud incomplete) while the second immunization after four weeks of the first immunization and the third immunization after four weeks of the second immunization were conducted without the use of adjuvants.

2.4 Sera

Immunized and non-immunized groups. Sera from immunized (n=49) and non-immunized (n=2) groups were collected two weeks post the final immunization. The non-immunized sera were collected from fish that were injected with PBS only. The fish were sedated using a mixture of Metacaine and Metomidate and stunned to death by a blow to the head. Blood was drawn from *Vena Caudalis* and directly transferred to blood collection tubes without additives (BD Vacutainer). The blood samples were placed at 4ºC overnight to allow blood coagulation. The coagulated blood was centrifuged at 2500xg (Allerga X – 15R Centrifuge, Beckman Coulter) for 15 minutes at 4ºC. The serum fraction was carefully extracted and stored at -20ºC in small aliquots for further analysis.

Non – vaccinated challenged group. Sera from this group were collected from survivals of fish post challenge with AL20460 (atypical *A. salmonicida*), 8546 (atypical *A. salmonicida*), 8752 (*V. anguillarum* O1) or *Pasteurella* spp. The fish had been challenged by bath, intraperitoneal (i.p.) injection or cohabitation. For bath challenge, the fish had been exposed to a bacterial concentration of $2x10^6$ bacteria/ml for 1 h. The cohabitation and i.p challenge experiments were carried out in tanks with i.p to cohabitant ratios of 1:1 or 1:2, respectively. Challenge against strains AL20460 (atypical *A. salmonicida*), 8546 (atypical *A. salmonicida*) and 8752 (*V. anguillarum* O1) had been performed by

cohabitation and i.p. injections of 50 µl of $1x10^3$ or $1x10^6$ bacteria/ml. For challenge against the *Pasteurella* spp., the fish were injected with bacterial suspensions of $5.1x10^8$ at 1/20, 1/50, 1/100 or 1/1000 dilutions in PBS. The non-challenged control sera were collected from non-challenged fish that were injected with PBS only.

Vaccinated challenged group. Sera were collected from survivals of vaccinated fish group that were challenged against AL20460 (atypical *A. salmonicida*), 8546 (atypical *A. salmonicida*) or 8752 (*V. anguillarum* O1). The fish were i.p. injected (before challenge) with vaccines listed in table 2. The vaccinated fish were marked with green (Trivalent), blue (Monovalent) or red (Alpha Marine Micro 3) fluorescent dyes. Non-vaccinated challenged fish injected with PBS were also included in the experiment and these were not marked. The bacterial concentration used for the challenge experiment was based on mortality profiles in challenge models of non-vaccinated fish (Rønneseth, 2015).

Table 2. Injectable vaccines used for vaccination of the lumpfish before challenge. Alpha Marine Micro 3 is a commercial vaccine developed for Atlantic cod (*Gadus morhua*) by Pharmaq AS, Norway.

Vaccine	Challenge	Content
	8546 (atypical *A. salmonicida*)	Atypical *A. salmonicida*
Monovalent	AL20460 (atypical *A. salmonicida*)	Atypical *A. salmonicida*
	8752 (*V. anguillarum* O1)	*V. anguillarum* O1
	8546 (atypical *A. salmonicida*)	Atypical *A. salmonicida, V. anguillarum* O1
Trivalent	AL20460 (atypical *A. salmonicida*)	& *Pasteurella* spp.
	8752 (*V. anguillarum* O1)	
AMM3	8546 (atypical *A. salmonicida*)	Atypical *A. salmonicida* and *V. anguillarum*
	AL20460 (atypical *A. salmonicida*)	(O2a & O2b)

2.5 Enzyme – Linked – Immunosorbent - Assay

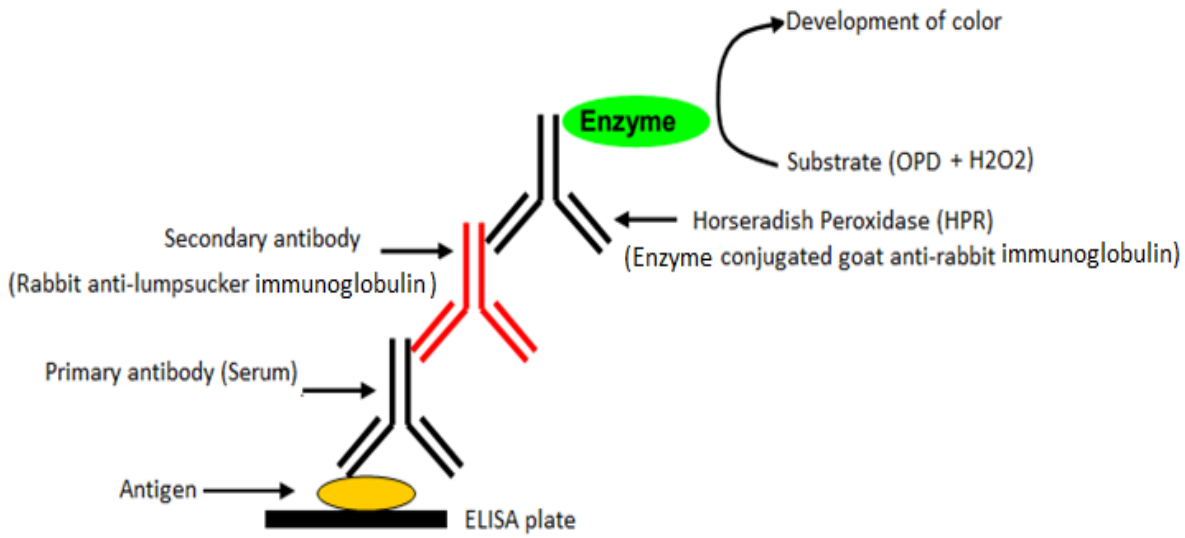


Figure 6. A diagrammatic description of the ELISA method used. Modified from: University of Bergen Fish Immunology Laboratory Journal.

The same ELISA procedure as shown in fig. 6 were employed for all groups. ELISA with a flat-bottomed 96 well microtiter plate in an 8x12 format (F96 MAXISORP NUNC-IMMUNO PLATE) was used to test for antibody levels in fish sera post immunization and challenge with or without vaccination. The ELISA was also used to test antibody cross-reactivity between strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) and also between strains 8752 (*V. anguillarum* O1) and AL-122 (*V. anguillarum* O2). Strain 8752 (*V. anguillarum* O1) was used for challenge, whereas AL-122 (*V. anguillarum* O2) was included as antigen in the vaccine and for antisera production.

First, the concentration of antigen needed for coating the ELISA wells was optimized. Concurrently, the effect of 3% skimmed milk blocking solution on antigen was also determined. For antigen optimization, 1 mg/ml of antigen from a stock solution was diluted in PBS – T to make five final concentrations of 10 µg/ml, 8 µg/ml, 6 µg/ml, 4 µg/ml and 2 µg/ml from each bacterium according to a dilution equation (Eq. 1). The antigen concentration that gave the highest response in terms of optical density (OD) at 492 nm was selected.

$$C1V1 = C2V2 \qquad \text{Eq. 1}$$

Where

C1 = concentration 1
C2 = concentration 2
V1 = volume 1
V2 = volume 2

For testing the effect of blocking solution on antigen, the 96 well ELISA plate was divided into two equal parts, one part with a blocking solution (200 µl per well) and the other part without a blocking solution. A volume of 200 µl PBS-T per well was added to the unblocked part. The wells were coated with 10 µg/ml of WB antigen (150 µl/well). The plates were covered with an empty microtiter plate, wrapped in a plastic and incubated at 4 ºC overnight. Excess unbound antigens were removed by washing with 200 µl PBS-Tween per well using a washer (Nunc-Immuno^{TM}Wash 12). The washing, covering and wrapping procedures were the same for all the steps. The antigen was not blocked with 3% skimmed milk when testing for antibody levels in immunized, non-immunized and challenged groups and also for cross reactivity test.

Serum was diluted in PBS – T; different sera dilutions listed in table 3 were used. A volume of 100 µl of serum diluted in PBS-T was loaded into the well in duplicates and incubated overnight at 4 ºC. The plates were washed and 50 µl of rabbit anti-lumpfish, a secondary antibody diluted 1/1000 in PBS-T, was added to each well. The rabbit

anti-lumpfish Ig was obtained from the fish immunology group at the University of Bergen. The plates were incubated for two hours at room temperature (RT) and after washing the plates, 50 µl horseradish peroxidase-conjugated goat anti-rabbit Ig (HPR, DAKO) diluted 1/1000 in PBS-T was added and incubated for 1 hour. Peroxidase substrate solution was prepared and 15 µl hydrogen peroxide (H_2O_2) (Sigma Aldrich) was added to the substrate solution just before loading the substrate into the wells. After final washing, 50 µl of peroxidase substrate solution was added to each well resulting in development of colored products. The reaction was stopped after six minutes by adding 50 µl 2.5N sulfuric acid (H_2SO_4) into each well. The plates were read by a microplate reader (Tecan) at 492nm. The final OD readings were used to compare between different groups.

Table 3. Dilution of sera used for detection of antibodies by ELISA

Test	Dilution
Antigen optimization	1/50 to 1/800 (twofold)
Specific antibodies (antibody titration) in immunized groups	1/50 to 1/25,600 (twofold)
Antibody cross reactivity	1/50
Antibody detection in challenged groups	1/200 & 1/800

2.6 Gel electrophoresis of proteins

Proteins from the sonicated WB antigens were separated using sodium-dodecyl sulfate – polyacrylamide gel electrophoresis (SDG-PAGE). The mini-PROTEAN Tetra cell casting module (Bio-Rad) was properly assembled and the space in-between the short and spacer (0.75mm) glass plates was filled with 12% resolving gel in a mixture with 5 µl Tetramethylethylenediamine (TEMED) (Sigma Aldrich) and 50ul 10% ammonium persulfate (APS). A little amount of deionized water (Milli-Q) was added on top of the gel. After pouring off the water, 4% stacking gel was added on top of the polymerized resolving gel. A comb (Mini-PROTEAN® Comb, 10-well, 1.0 mm, 44 µl) was put on top of the stacking gel to create wells for sample and standard proteins. The stacking gel was

allowed to polymerize at RT for 45 minutes. The comb was removed from the casting frame (Mini-PROTEAN® Bio-Rad). The gel together with the glass plates were fastened into a Mini-PROTEAN® Tetra Electrode Assembly and immersed into a buffer tank (Mini-PROTEAN® Tetra cell, Bio-Rad) filled with 1xTris-Glycine-Sodium-dodecyl-sulfate (TGS, pH 8.3) buffer. A concentration of 2 mg/ml freeze-dried sonicated WB from strains 8752 (*V. anguillarum* O1), ALL-122 (*V. anguillarum* O2), AL-20460 (atypical *A. salmonicida*), 8546 (atypical *A. salmonicida*), 8652 (*V. ordalii*), *Pasteurella* spp. and *P. skynesis* were prepared. Equal volumes of the sonicated WB sample and gel-loading buffer, also called 2xLaemmli concentrate, containing 2-mercaptoethanol were mixed and boiled for 5 minutes at 98ºC using a thermo-mixer (Eppendorf). For silver stain, 10 µl unstained low range SDG-PAGE standard (#161-0314, Bio-Rad) diluted 1/20 in the gel-loading buffer was used, whereas 5 µl Precision Plus™ Kaleidoscope™ pre-stained standard (#161-0324, Bio-Rad) was used in gels for immunoblotting. A volume of 10 µl from each sample was pipetted and loaded into their respective wells in the gel. The buffer tank was covered with a cell lid attached to power cables (Bio-Rad) and 190 volts of electrical current was passed across the gel for 45 minutes.

2.7 Silver staining of proteins

After protein separation on the SDS-PAGE, the proteins were fixed for 20 minutes with a fixative solution. The gel was washed 2x10 minutes in deionized water and stained with silver-staining technique (Switzer et al., 1979) for 40 - 45 minutes until clear bands were formed. The staining solution was replaced by 5% acetic acid and the gel with the protein bands was photographed. The molecular weight of the sample proteins was estimated according to a protocol for molecular weight estimation by the Bio-Rad (BIO-RAD, 2015). The relative distances (Rf) of the standard and unknown proteins were determined using eq. 2. Using the values for the standard proteins, a linear standard curve was developed by plotting the logarithm of the molecular weight (logMW) of the standard proteins on the y – axis and its Rf value on the x – axis. Then, the molecular weight of the sample was estimated by interpolating the Rf values of the sample into the x variable of the linear formula obtained from the standard curve.

$$Rf = \frac{\text{Migration distance of the protein (cm)}}{\text{Migration distance of the dye front (cm)}}$$ Eq. 2 (BIO-RAD, 2015)

2.8 Western blot

The protein gel, a nitrocellulose membrane (0.45µm) (Bio-Rad), blot absorbent chromatography papers (Whatman®) and foam pads were soaked in transfer buffer for 30 minutes. The protein gel and the nitrocellulose were tightly placed together and sandwiched in between the moist blot absorbent chromatography papers assembled in a gel holder cassette as described by Garfin (2003) (fig. 7). The cassette was submerged in transfer buffer in a vertical position inside a buffer tank mounted with positive and negative electrodes at the opposite sides of the tank. The tank was covered with a lid attached to electrical cables. Identical copies of the proteins in the same pattern as in the gel were copied and immobilized on the nitrocellulose membrane by applying a 100 volt electrical current across the gel for 1 hour in the presence of ice. After the electrophoretic transfer of the proteins, the membrane was removed and unbound protein sites were blocked by incubating the membrane in Tris buffered saline (TBS) with 3% skimmed milk for 1 hour at RT. The membrane was washed 2x5 minutes in TTBS and the same washing procedure was applied in every step of the immuno-detection (fig. 8). The protein blot was probed with specific primary antibody by incubating the membrane for overnight at 5ºC in antiserum (collected from immunized groups) diluted 1/100 in TTBS with 1% skimmed milk (antibody dilution buffer). All the immune-detection procedures after this step were conducted at RT. The blot was probed for the second time with rabbit anti-lumpfish Ig (secondary Ig) and the third time with enzyme conjugated goat anti-rabbit Ig for incubation period of 1 ½ hours each. Both anti-lumpfish Ig and enzyme conjugated Ig were diluted 1/500 and 1/1000 in the antibody dilution buffer, respectively. After the final washing, the membrane was placed in TBS for 5 minutes just before imaging the blots. The membrane was immersed for 5 minutes in a mixture consisting of equal volumes of Clarity ECL Western Blotting substrates (Peroxide and *luminescence*

substrates) (Bio-Rad). The best blot was considered by shooting and comparing several blot images at different exposure times using Molecular Imager® ChemiDoc™ XRS system (Bio Rad). The presence of cross reactivity between the samples was checked. The molecular weight of the protein bands was estimated from the Kaleidoscope Pre-stained Standards (#161-0324, Bio-Rad).

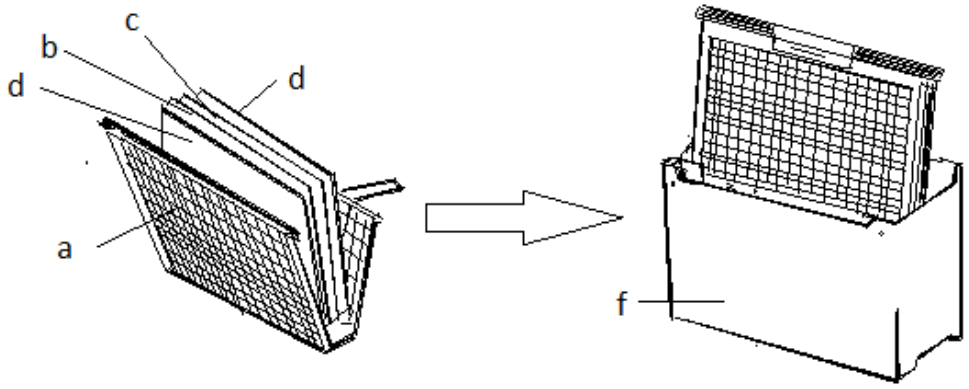


Figure 7. A protein tank electro-transfer device in an exposed view (Garfin, 2003). The gel (b) and the membrane (c) in between the moist blot absorbent filter papers and foam pads (d) were held by the cassette (a). The cassette (e) was vertically placed in the tank (f) filled with the transfer buffer.

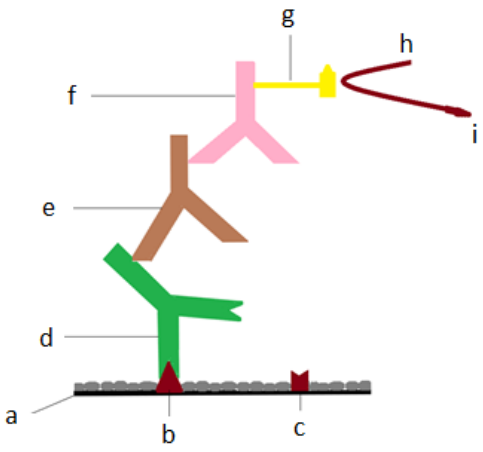


Figure 8. A diagrammatic description of immuno-detection by western blot modified from Bio-Rad Laboratories, Inc. (BioRad, 2015). The lumpfish (primary) antibody (d) was specifically bound to the target protein (b) fixed in the nitrocellulose membrane (a). Free sites in the membrane were blocked (c) with a blocking solution. The primary antibody was probed with rabbit anti-lumpfish (secondary) antibody (e). The specific antibody was detected with the help of a goat anti-rabbit (tertiary) antibody (f) conjugated to enzyme (g), which acts on the substrate (h) to give a detectable product (i) (BioRad, 2015).

2.9 Bradford protein assay for determination of purified lumpfish IgM concentration

The concentration of purified lumpfish IgM was determined by generating a standard curve according to the Bradford Protein Assay (Bradford, 1976). The purified IgM was obtained from the fish immunology group at the University of Bergen. Bovine Serum Albumin (BSA) (Bio-Rad Laboratories, Inc.) was used as a standard protein. Initial concentration of 1.08 mg/ml of BSA was used to make four final concentrations by pipetting 50 µl, 100 µl, 200 µl and 300 µl of the standard protein into 450 µl, 400 µl, 300 µl and 200 µl of PBS making a final volume of 500 µl each, respectively. A solution containing 1 part Coomassie Brilliant Blue G-250 (Imperial Chemical Industries) and four parts distilled water (MilliQ) was also prepared. Four 5 ml test tubes of the known samples including two separate test tubes of the unknown and blank samples were filled with 1 ml of the solution. A 100 µl of the standard protein and unknown samples was

pipetted and added into their respective test tubes. PBS was added into the blank sample. After mixing and incubating at room temperature for 5 minutes, the samples were transferred to cuvettes and measured by a spectrophotometer at 595 nm to obtain OD. The OD's of the blank samples were subtracted from the OD's of the standard protein and the unknown samples. A standard curve was generated by plotting sample OD's in the y-axis against the concentrations of the standard protein in the x – axis. The concentration of the purified IgM was calculated from the standard curve. The purified IgM with a known concentration was used as reference IgM for the determination of protein content in all immunized fish sera.

2.10 Single Radial Immunodiffusion Assay for determination of total serum IgM

SRID was used to quantify the total IgM concentrations in in sera of immunized and non-immunized groups in reference to the standard proteins. The procedures of the SRID assay were the same for all the experiments. Agarose gel was prepared by mixing 0.335 g agarose 1% (Lonza Group Ltd) and 0.67 g 2% polyethylene glycerol (PEG) 6000 (Sigma-Aldrich, CO.) in 33.33 ml PBS (pH 7.2, Lonza Group Ltd). The mixture was boiled in a microwave oven (Whirlpool) at 930 watt until the crystals were completely homogenized. The gel was cooled down to 55ºC and 134.11 µl rabbit anti-lumpfish IgM diluted 1/250 in PBS (pH 7.2, Lonza Group Ltd) was incorporated into the gel. Care was taken to avoid denaturation of Ig by the heated mixture. A thin transparent and flexible plastic plate, GelBond®film (Pharmacia LKB Biotechnology AB) was used to support the agarose gel. The film was laid down on to a clean rectangular 13.5x23x0.4 cm (143.1 cm^3) hard plastic pre-warmed in an incubator (Termarks AS) at 55 – 60 ºC. The gel was carefully poured onto the hydrophilic side of the film, making sure that the gel was evenly spread. After the gel was polymerized for ca. 15 minutes at RT, small circular wells (4 mm in diameter) were cut on the gel surface by punching it with a revolving leather belt hole puncher (POWERFIX®).

The purified lumpfish IgM was used as a reference for generating a linear standard curve. Six concentrations of the purified IgM ranging from 160 ng/µl to 5 ng/µl twofold dilutions

were prepared. A standard curve was included for each group. The standard proteins and the sera from the immunized (n=10) and non-immunized groups (n=2) were diluted at 1/20 in PBS (pH 7.2). Then, 5 µl of the samples including the standard proteins were pipetted, loaded in duplicates into their respective wells and incubated in a moist chamber for 48 hrs. Afterwards, a 140x270mm filter paper (Struers KEBO LAB AB) moistened with PBS was carefully laid down onto the agarose gel. Additional five dry filter papers were put on top of the moistened filter paper; the gel was pressed by placing 864 g of weight on top of the dry filter papers for 5 minutes. The filter papers, including the moistened one, were carefully removed from the gel and the gel was immersed in PBS for 15 minutes. The PBS was removed from the gel and the pressing procedure was repeated followed by washing in PBS for 15 minutes. After the second washing, the gel was again pressed with a moistened and five dry filter papers for five minutes and dried in an incubator at 40 ºC for 1 hr. The gel was stained with a Coomassie blue solution for 15 minutes and unstained by Coomassie destaining solution. Circular precipitins were observed around the well. The diameter of the precipitins from the standard proteins were measured using Adobe Illustrator CS5 software and plotted against the concentrations of the purified IgM to make a standard curve with a linear equation and R^2 value. The linear equation (Eq. 3) (Hazewinkel, 2001) from the standard curve was used for quantification of IgM concentrations in both immunized and non-immunized groups. The y variable was represented by the diameter of the precipitin in millimeter (mm) and x variable by IgM concentration in ng/µl. To evaluate the IgM concentration, x variable was substituted with the diameter of the precipitin. The final x values were multiplied by the dilution factor to find out the serum IgM concentration in mg/ml. The total IgM concentrations in both the immunized and unimmunized groups were compared.

$$y = ax + b \qquad a \neq 0$$ Eq. 3 (Hazewinkel, 2001)

Where, y = diameter of a ring in mm (dependent variable)
x = IgM in ng/µl (independent variable)
b = a constant or value of a dependent variable when x = 0 (y 0 incercept)
a = a constant or coefficient of independent variable (slope)

2.11 Statistics

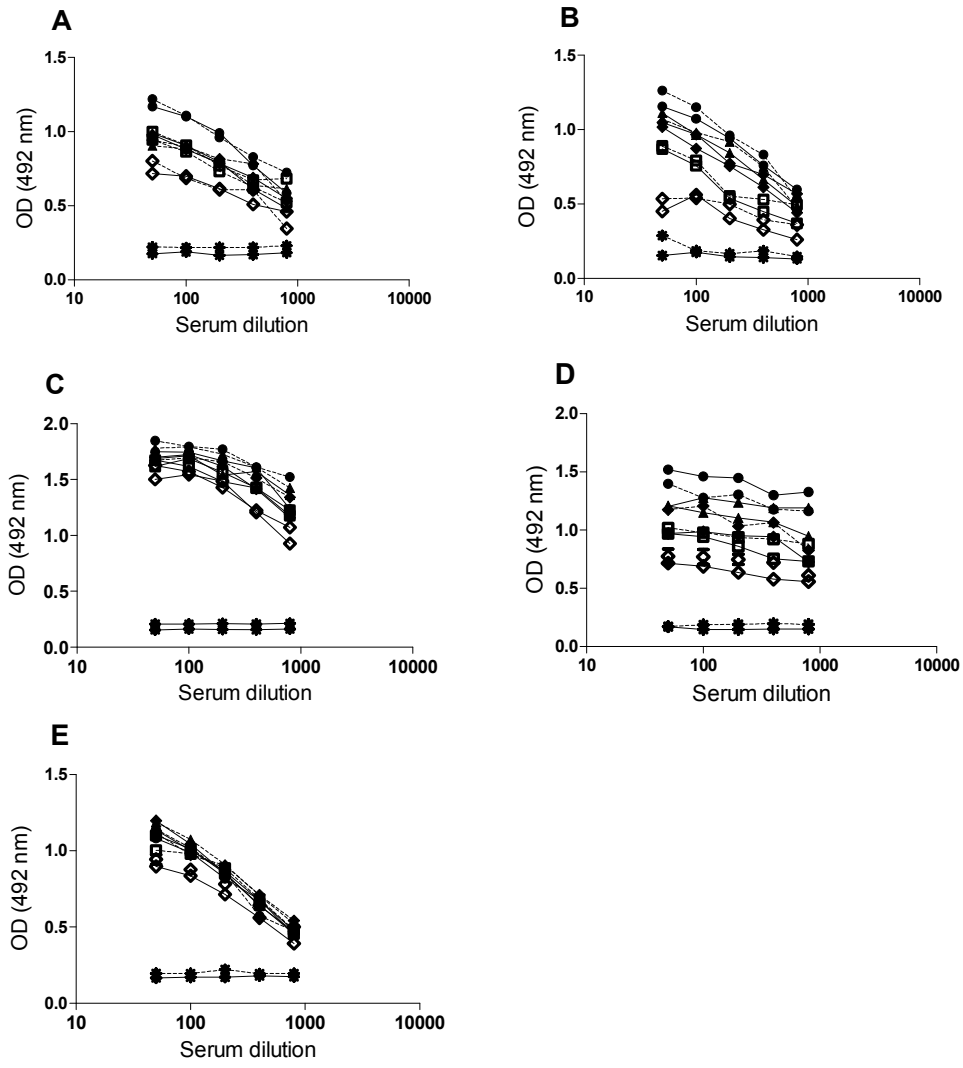
Data were statistically analyzed by use of GraphPad prism version 5.00 for windows (GraphPad, 2007) or R program for statistics (R Core, 2014). GraphPad Prism was used to make antibody titration curves. Shapiro-Wilk test (Royston, 1982) and Quantile-Quantile plot (Q-Q plot) (Becker et al., 1988) were performed to test for normality of the data using R program for statistics (R Core, 2014). Both student t-test and a non-parametric Wilcoxon-Mann Whitney test for independent samples were used for comparison of means or medians between groups due to non-Gaussian distribution in some groups. Comparisons within or between groups were described by boxplots. A horizontal line inside the boxplots represents the median. The bottom of the box is the first quartile, which is less than or equal to 25% of the data, whereas the top of the box is the third quartile, which is less than or equal to 75% of the data. The vertical upper and lower whiskers that extend outside the box are highest and lowest data values, respectively. Data points elsewhere are outliers (McGill et al., 1978). Statistical inference was made based on p values, i.e. observations were considered similar when $p > 0.05$ but different when $p < 0.05$.

3. RESULTS

3.1 ELISA

3.1.1 *Antigen optimization and the effect of blocking solution on antibody response*.

After a few seconds of adding the OPD substrate to the wells, development of brownish colour was observed (not shown), which increased in strength until the reaction was stopped at 6 minutes. The OD (492 nm) values were proportional to the amount or strength of colour developed, i.e. strong colours were formed at wells with high antigen concentrations and low serum dilutions for all groups or vice versa. High antibody response with strong colour was observed at 10, 8, or 6 µg/ml of antigen (fig. 9). The optimal antigen dilution that could be used to detect specific antibodies was recorded at 10 µg/ml of antigen. Moreover, blocking had no effect on antibody response ($p>0.05$).



●10 μg/ml; ▲ 8 μg/ml; ◆6 μg/ml; □4 μg/ml; ◇2 μg/ml; ✳ blank

Fig. 9. Antibody dilution curves at different antigen concentrations in groups immunized against formalin inactivated WB antigens of strains 8546 (atypical *A. salmonicida*) (A), AL20460 (atypical *A. salmonicida*) (B), AL122 (*V. anguillarum* O2), (C), *Pasteurella* spp. (D), and 8657 (*V. ordalii*). The ELISA plates were coated with 150 μl freeze-dried, sonicated WB Ags. The optimized antigens were homologous, except for group AL122 (C), which was tested against a heterogeneous antigen from strain 8752. Continuous and broken lines indicate for the use and without the use of 3% skimmed milk blocking solution in the blocking step, respectively.

3.1.2 Response to immunization.

The presence of antigen specific antibody two weeks post-immunization was determined by ELISA; the results were described by a sigmoidal curve in a dose-response relationship, where OD (response) is a function of the logarithm of serum dilution (dose).The level of antigen – antibody reactivity was different between groups; there was also large individual variation within groups (fig. 10), i.e. some fish were high responders, while others were low responders. The specific antibody response in each group was compared at 1/400 serum dilution; antiserum from fish immunized with *Pasteurella* spp. contained the highest specific antibodies followed by AL122 (*V. anguillarum* O2), AL20460 (atypical *A. salmonicida*), 8546 (atypical *A. salmonicida*) and 8657 (*V. ordalii*) (fig. 11, table 4). However, no significant difference ($p>0.05$) in antibody level was observed for antiserum from fish immunized against strain 8657 (*V. ordalii*).

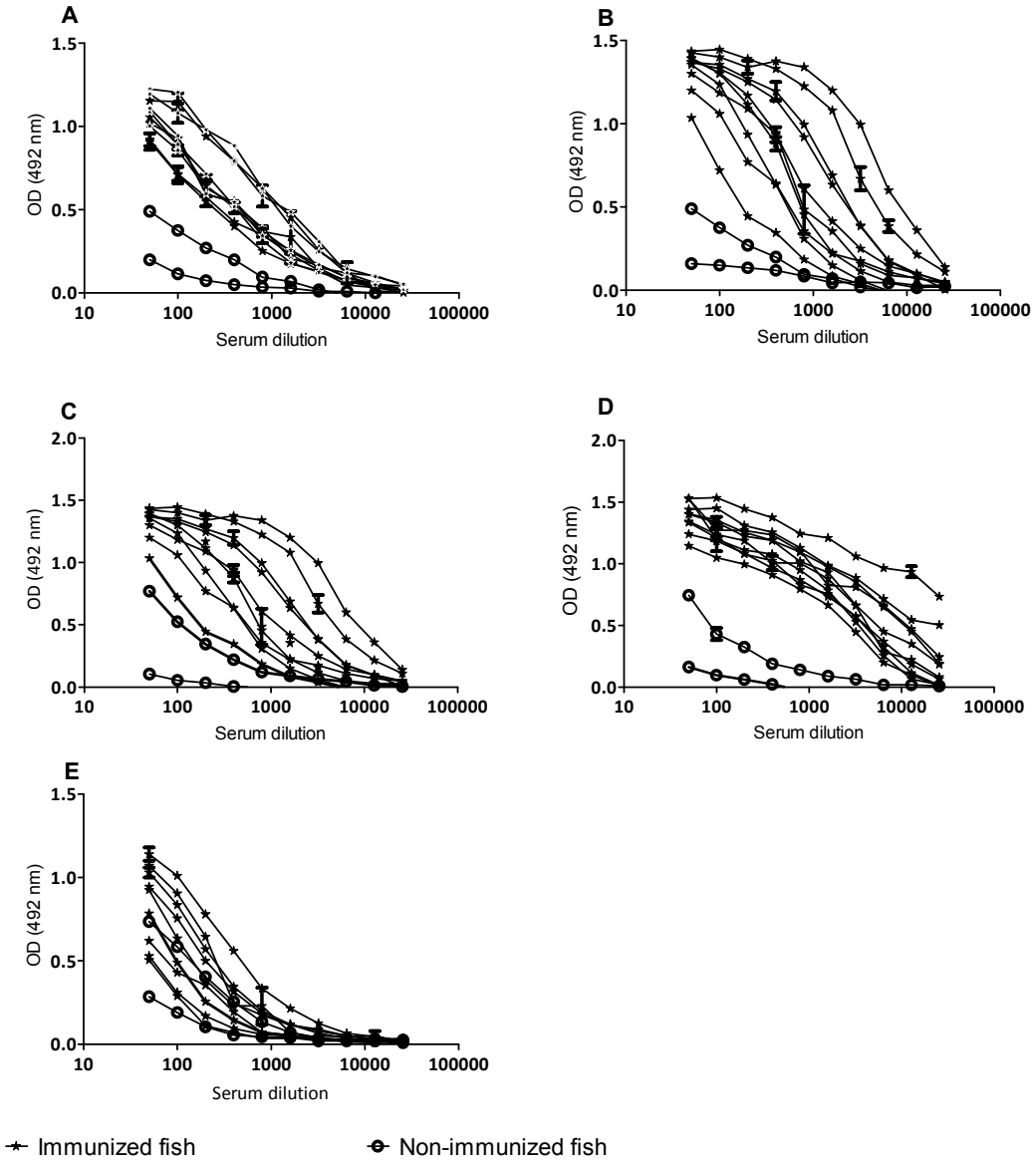


Fig. 10. Serum antibody dilution curves for the different immunized groups quantified in ELISA. A) Antiserum: 8546 (atypical *A. salmonicida*); B) Antiserum: AL20460 (atypical *A. salmonicida*); C) Antiserum: AL122 (*V. anguillarum* O2); D) Antiserum: *Pasteurella* spp.; E) Antiserum: 8657 (*V. ordalii*). Homologous sonicated WB cells were used as coating antigen in ELISA with exception to antiserum to AL122 (*V. anguillarum* O2), which was tested using a heterogeneous antigen from strain 8752 (*V. anguillarum* O1).

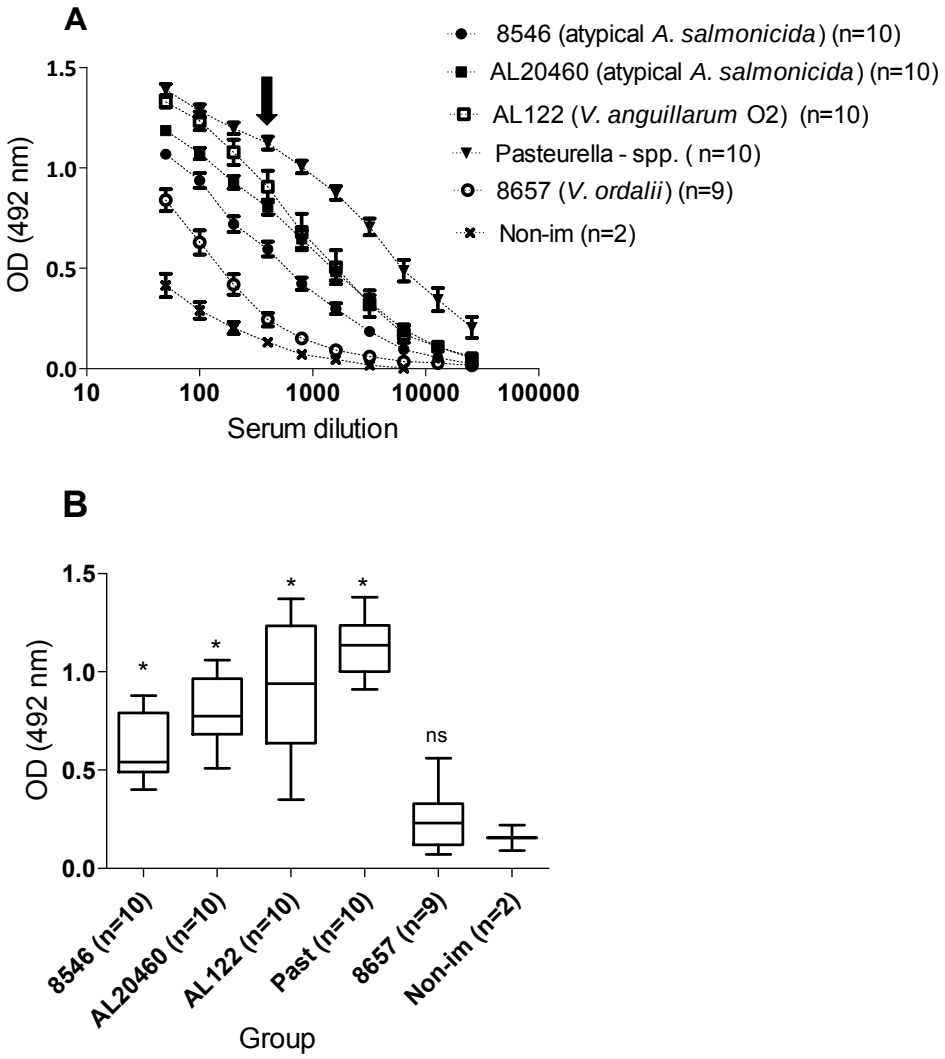


Fig. 11. Mean antibody titration curves for each group of immunized fish (A) and comparison of specific antibody levels between groups at 1/400 serum dilution (B) by ELISA. The arrow in fig. 11A indicates for mean specific antibody levels at 1/400 serum dilution. The tests were conducted using homologous sonicated WB cells as antigens in ELISA. Antiserum to AL122 was tested using a heterogeneous from strain 8752 (*V. anguillarum* O1). Individual serum dilution curves are shown in fig. 10. *, $p<0.05$; Non-im, non-immunized control.

Table 4. Mean specific antibodies at 1/400 serum dilution for immunized and non – immunized groups.

Group	**N**	**Mean OD (492 nm) ± SD**	**CV**
8546 (atypical *A. salmonicida)*	10	0.60 ± 0.16	0.28
AL20460 (atypical *A. salmonicida*)	10	0.80 ± 0.17	0.21
AL122 (*V. anguillarum* O2)	10	0.94 ± 0.33	0.34
Pasteurella – spp.	10	1.12 ± 0.15	0.13
8657 (*V. ordalii*)	10	0.25 ± 0.15	0.61
Non – immunized control	2	0.16 ± 0.09	0.59

3.1.3 Cross-reactivity test.

Antibody cross – reactivity between strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) and between strain AL122 (*V. anguillarum* O2) and 8752 (*V. anguillarum* O1) were tested by ELISA. The antisera to 8546 and AL20460 were reactive to one another, showing significantly higher antibodies when compared to non-immunized control serum (fig. 12, table 5). The amount of antibody response to the heterogeneous and homogeneous antigens was similar ($p>0.05$).

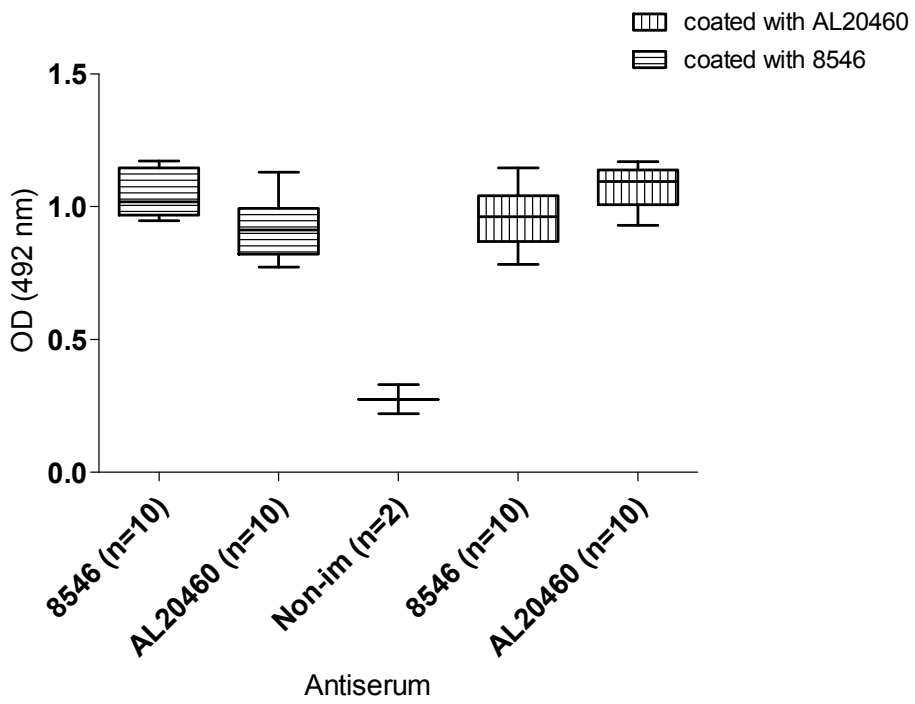


Fig. 12. Analysis of cross reactive antibodies in antisera raised against strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) to freeze dried sonicated WB used as antigens in ELISA. Serum diluted 1/50 in PBS – T. Serum diluted 1/50.

Table 5. Mean antibody levels of antisera (1/50 dilution) when tested for cross-reactivity with heterogeneous freeze-dried sonicated WB used as Ag in ELISA.

Antisera	**Coating antigen**	**N**	**Mean OD (492 nm) ± SD**
8546 (atypical *A. salmonicida*)	8546	10	1.04 ± 0.09
	AL20460	10	0.92 ± 0.11
AL20460 (atypical *A. salmonicida*)	8546	10	0.96 ± 0.11
	AL20460	10	1.08 ± 0.08
Non-immunized control	8546/AL20460	2	0.28 ± 0.08

Similarly, antiserum to strain AL122 (*V. anguillarum* O2) had Abs cross reactive to freeze-dried sonicated WB antigens from strain 8752 (*V. anguillarum* O1). Elevated levels of antibodies were detected to either homogeneous or heterogeneous antigens compared to the non-immunized control serum (fig. 13, table 6). The level of antibodies to homogeneous and heterogeneous antigens were the same ($p>0.05$). However, high levels of antibodies were also detected in one of the non-immunized control sera.

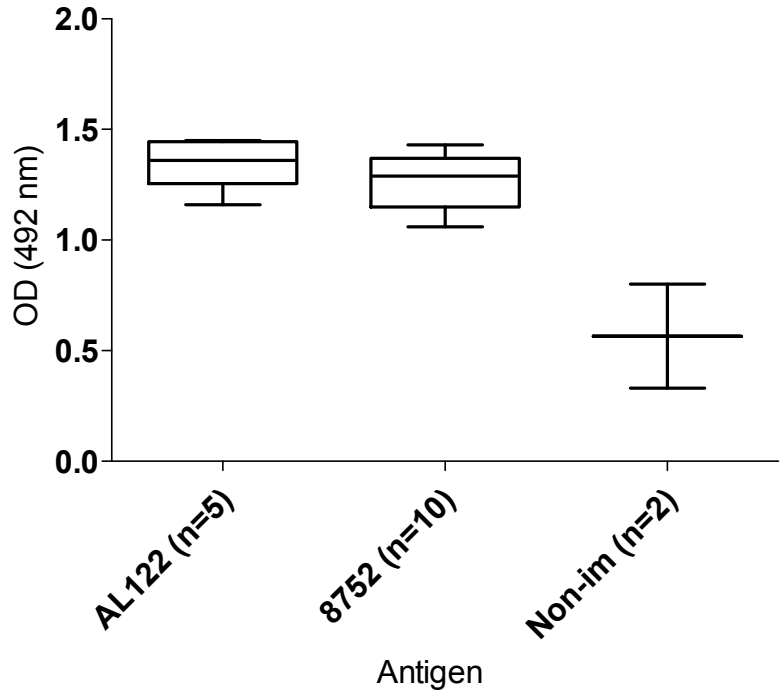


Fig. 13. Analysis of cross-reactive antibodies by ELISA in antiserum raised against strain AL122 (*V. anguillarum* O2). For cross-reactivity test, the ELISA wells were coated with heterogeneous sonicated WB cells of strain 8752 (*V. anguillarum* O1) and compared to the level of antibodies to the homologous antigen in ELISA. Serum diluted 1/50; ns, $p>0.05$.

Table 6. Mean antibody levels of antiserum to strain AL122 (*V. anguillarum* O2) (1/50 dilution) when tested for cross-reactivity with heterogeneous freeze-dried sonicated WB used as antigen in ELISA.

Antiserum	Coating antigen	N	Mean (OD) ± SD
AL122 (*V. anguillarum* O2)	AL122 (*V. anguillarum* O2)	5	1.35 ± 0.12
	8752 (*V. anguillarum* O1)	5	1.27 ± 0.13
Non-immunized control	8752/AL122	2	0.57 ± 0.33

3.1.4 *Response to non-vaccinated challenged group.*

Sera from challenge survivals were collected 35, 49, 43 and 11 days post challenge against strains 8546 (atypical *A. salmonicida*), AL20460 (atypical *A. salmonicida*), 8752 (*V. anguillarum*) and *Pasteurella* spp., respectively. ELISA analysis showed that all sera from all different challenge groups had low antibody levels. Individual variation in antibody levels was also observed in all sera samples analysed by ELISA.

No significant antibody levels were detected in sera of fish survivals that were challenged against strain 8546 (atypical *A. salmonicida*) by bath, cohabitation or i.p. compared to the non-challenged control serum ($p > 0.05$) (fig. 14). For fish challenged by cohabitation, only sera collected from fish that were challenged by cohabitation in a 1 to 1 ratio to i.p. were analysed for antibody response.

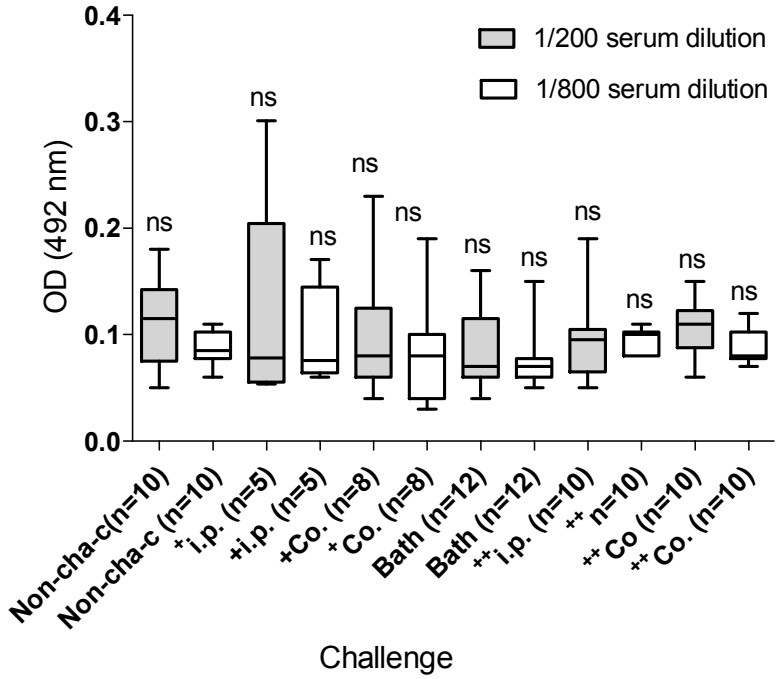


Fig. 14. Antibody levels detected by EILSA in sera of non-vaccinated fish 35 days post challenge against strain 8546 (atypical *A. salmonicida*) by different challenge exposures (bath, i.p. or cohabitation). Coating antigen: sonicated WB cells of strain 8546 (atypical *A. salmonicida*). Non-cha-c, non-challenged control; i.p., intraperitoneal injection; Co., cohabitation; +, 10^6 bacteria/ml; ++, 10^6 bacteria/ml.

Higher antibody levels were detected in sera from fish challenged with strain AL20460 (atypical *A. salmonicida*) by cohabitation ($p<0.05$) compared to the non-challenged control serum (fig 15). However, no significant antibodies were found in sera from fish challenged by i.p. compared to in sera of the non-challenged control group ($p>0.05$).

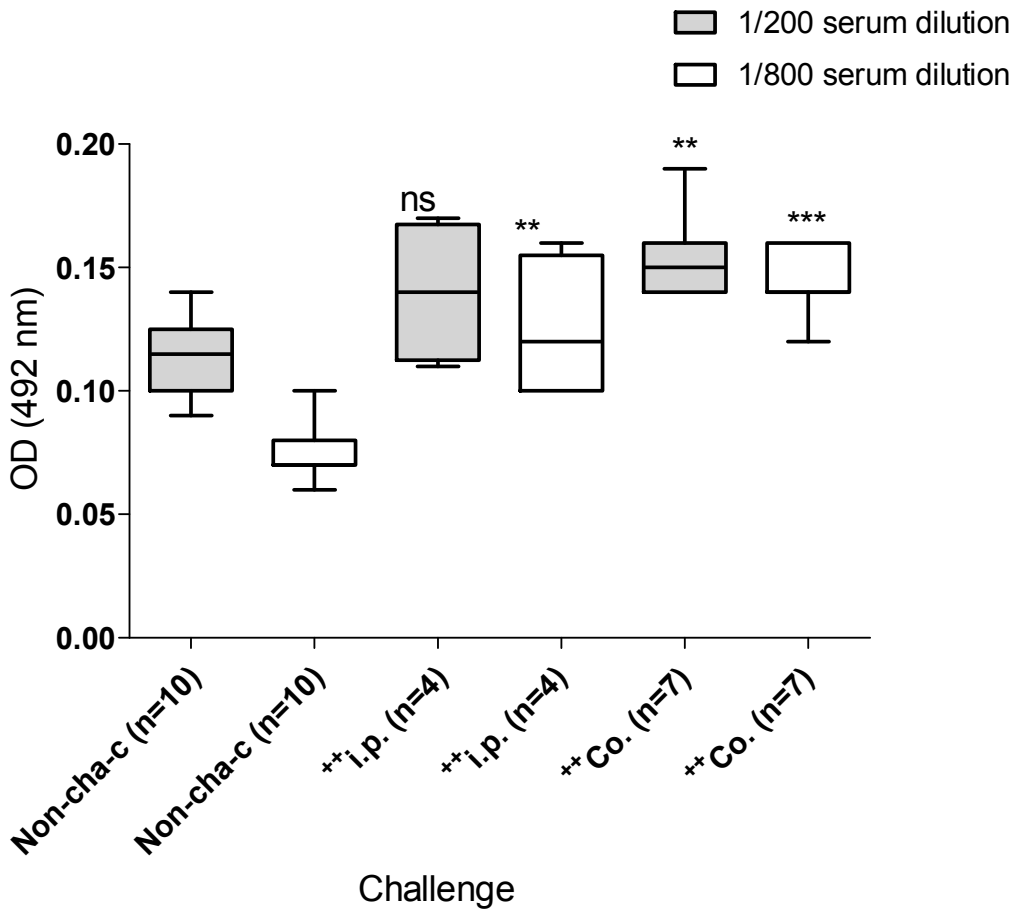


Fig. 15. Antibody levels detected by ELISA in sera of non-vaccinated fish 49 days post challenge with strain AL20460 (atypical *A. salmonicida*) by different challenge exposures (i.p. or cohabitation). Coating antigen: sonicated WB cells of strain AL20460 (atypical *A. salmonicida*). **, $p<0.01$; ***, $p<0.001$.

Challenge with strain 8752 (*V. anguillarum* O1) by i.p. or cohabitation revealed the presence of higher amount of antibodies compared to the non-challenged control group ($p<0.05$) (fig. 16). Highest Antibody levels were obtained in sera collected from i.p. challenged survivals, but only two fish survived from i.p. challenged group. The cohabitation challenge induced higher antibody levels than the bath challenge ($p<0.001$). In fact, the bath challenge did not induce significant antibody levels compared to the non-challenged control group ($p>0.05$).

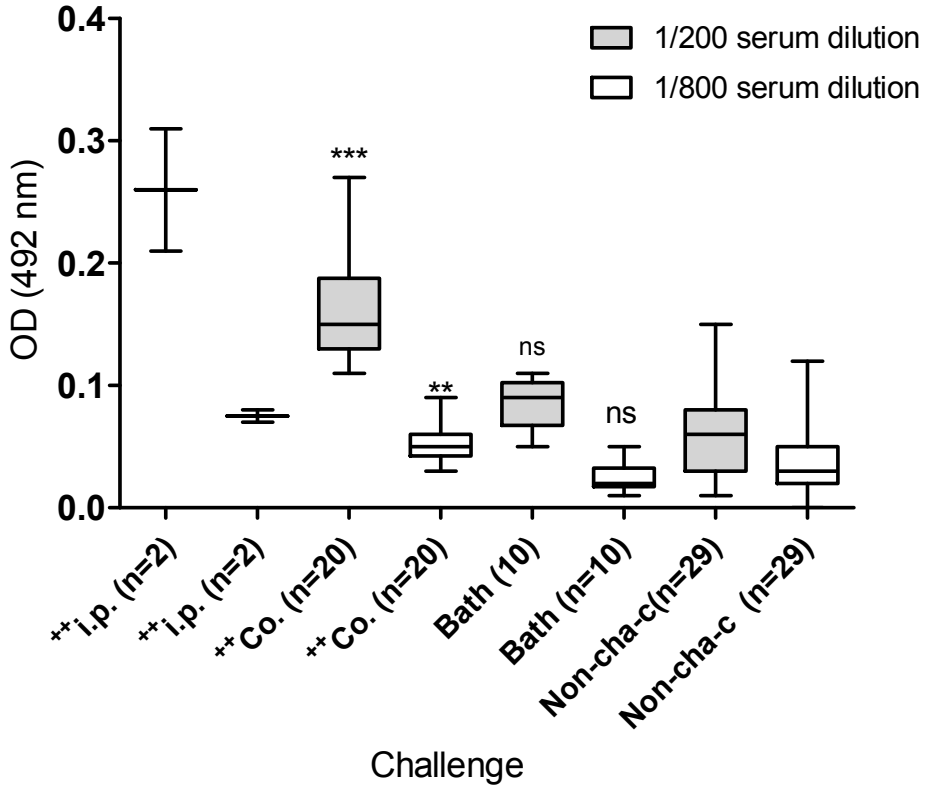


Fig. 16. Antibody levels detected by ELISA in sera of non-vaccinated fish 43 days post challenge with strain 8752 (*V. anguillarum* O1) by different challenge exposures (bath, i.p or cohabitation). Coating antigen: sonicated WB cells of strain 8752 (*V. anguillarum* O1).

Challenge with *Pasteurella* spp. was carried out by i.p. injection or cohabitation challenge at different bacterial concentrations, but only few from those challenged by cohabitation survived. Sera were analysed for the presence of specific antibody response 11 days post challenge. All sera from fish challenged with different bacterial concentrations contained higher antibody levels than sera from the non-challenged control group ($p<0.001$) (fig. 17). The same antibody levels were detected in sera from fish challenged with 1/50, 1/100 and 1/1000 bacterial dilutions ($p>0.05$). However, sera from fish challenged with 1/20 bacterial dilution contained the lowest antibody level ($p<0.05$).

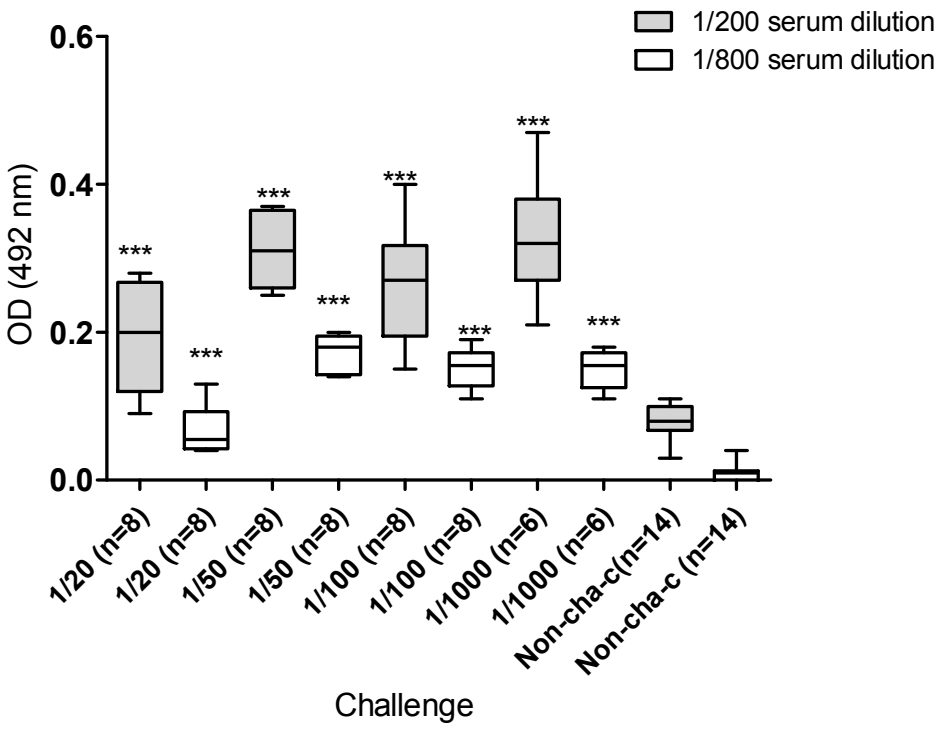


Fig. 17. Antibody levels detected by ELISA in sera of non-vaccinated fish 11 days post challenge with *Pasteurella* spp. through cohabitation by different doses of bacteria. Coating antigen: sonicated WB cells of *Pasteurella* spp.

3.1.5 Response to vaccinated challenged group

Sera from vaccinated challenged groups were analysed by ELISA for antibody response post challenge with strain 8546 (atypical *A. salmonicida*) and post challenge with strains AL20460 (atypical *A. salmonicida*) and 8752 (*V. anguillarum* O1). All sera collected from the survivals of vaccinated and non-vaccinated challenged (i.p.) groups revealed significantly higher antibody levels compared to the non-challenged control group ($p<0.001$). However, the antibody levels in sera of vaccinated challenged groups was much higher than that of the non-vaccinated challenged (i.p.) group ($p<0.001$).

The amount of serum antibody in sera from fish 43 days post challenge with strain 8546

(atypical *A. salmonicida*) were similar between fish that were treated with trivalent and AMM3 vaccines (p>0.05) (fig. 18). However, antibody levels in sera from fish vaccinated with monovalent vaccines was lower compared to sera from fish treated with trivalent vaccine (p<0.05).

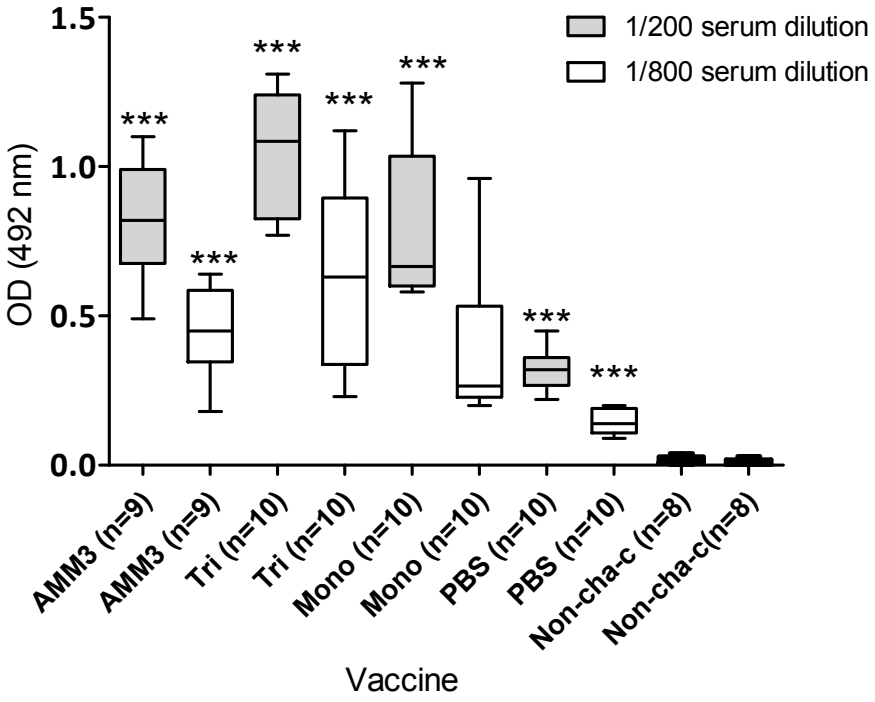


Fig. 18. Serum antibody levels in fish treated with different vaccines (AMM3, trivalent, or monovalent) 43 days post challenge (i.p.) with strain 8546 (atypical *A. salmonicida*). Coating antigen: sonicated WB cells of strain 8546 (atypical *A. salmonicida*). AMM3: Alpha Marine Micro 3; Tri: trivalent; Mono: monovalent; PBS: non-vaccinated (placebo) challenged; Non-cha-c: non-vaccinated non-challenged control group.

The antibody levels in fish treated with monovalent and AMM3 vaccines were similar (p>0.05) 31 days post challenge with strain AL20460 (atypical *A. salmonicida*), but sera from fish vaccinated with trivalent vaccine had slightly lower antibody levels compared to sera from fish treated with monovalent vaccine (p<0.05) (fig. 19). The trivalent and the commercial AMM3 vaccines against atypical furunculosis induced better antibody

response 43 days post challenge with strain 8546 (atypical *A. salmonicida*) than 31 days post challenge with strain AL20460 (atypical *A. salmonicida*) ($p<0.05$).

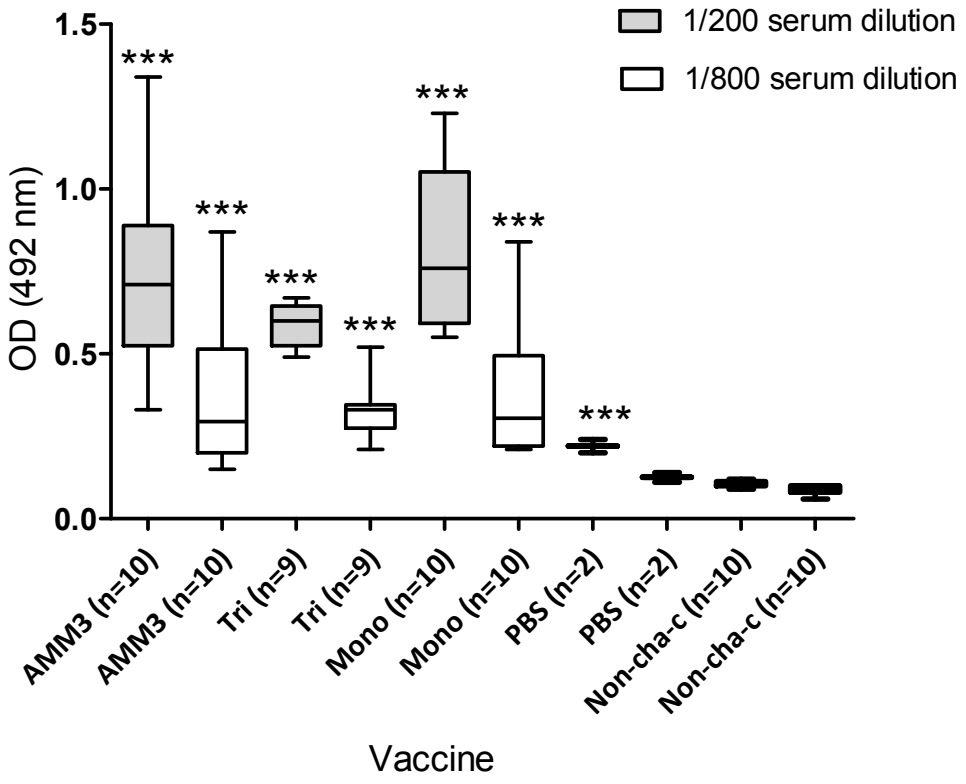


Fig. 19. Serum antibody levels detected by ELISA in fish treated with different vaccines (AMM3, trivalent, or monovalent) 31 days post challenge (i.p.) with strain AL20460 (atypical *A. salmonicida*). Coating antigen: sonicated WB cells of strain AL20460 (atypical *A. salmonicida*).

Fish challenged with 8752 (*V. anguillarum* O1) had been treated only with monovalent and trivalent vaccines; similar antibody levels were detected between sera from fish treated with these vaccines ($p>0.05$) (fig. 20).

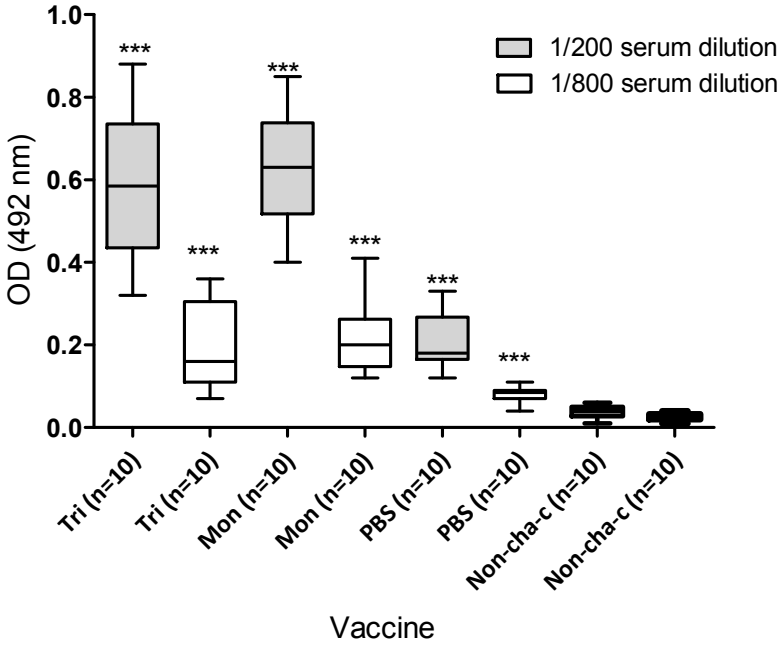


Fig. 20. Serum antibody levels detected by ELISA in vaccinated fish 31 days post challenge (i.p.) with strain 8752 (*V. anguillarum* O1). Coating antigen: sonicated WB cells of strain 8752 (*V. anguillarum* O1).

3.2 SDS – PAGE analysis of proteins

Fig. 21 shows a standard curve of low range SDS – PAGE standard protein and silver stained SDS – PAGE of different sonicated WB antigens. A wide range of protein bands of low and high molecular masses across the SDS – PAGE were observed. The size of protein band in each sample was estimated using the linear equation of the standard curve for the standard proteins (fig. 21A). All strains with exception to strain AL122 (*V. anguillarum* O2) had several major and minor protein bands visible across the SDS-PAGE (fig. 21B). Strain AL122 (*V. anguillarum* O2) failed to produce a single protein band in the SDS-PAGE (fig. 21B, lane 2).

The protein profile of each strain varied in band strength, and it appears the two atypical *A. salmonicida* strains (8546 and AL20460) showed slight differences (fig 21B, lanes 4 & 6). Unlike strain 8546 (atypical *A. salmonicida*), strain AL20460 (atypical *A. salmonicida*) possessed three clear bands at ca. 37, 40 and 45 kDa and a weak band at ca. 50 kDa (fig. 21B, lane 6). However, both strains shared similar proteins of high molecular weight (HMW) > 63 kDa protein and low molecular weight (LMW) < 32 kDa protein.

P. skyensis and *Pasteurella* – like spp. also displayed different protein profiles with major protein bands of ca. 37 and 35 kDa, respectively (fig. 21B, lanes 1 & 3).

Strains 8752 (*V. anguillarum* O1) and 8657 (*V. ordalii*) showed similar LMW protein profiles with prominent bands of ≤ 14.5 kDa. However, both strains had different molecular masses of major proteins at ca. 38 kDa for strain 8752 (*V. anguillarum*) and 36 kDa for strain 8657 (*V. ordalii*) (fig. 21B, lanes 5 & 7). Moreover, strain 8657 (*V. ordalii*) displayed several closely spaced bands above its major protein band of 36 kDa protein.

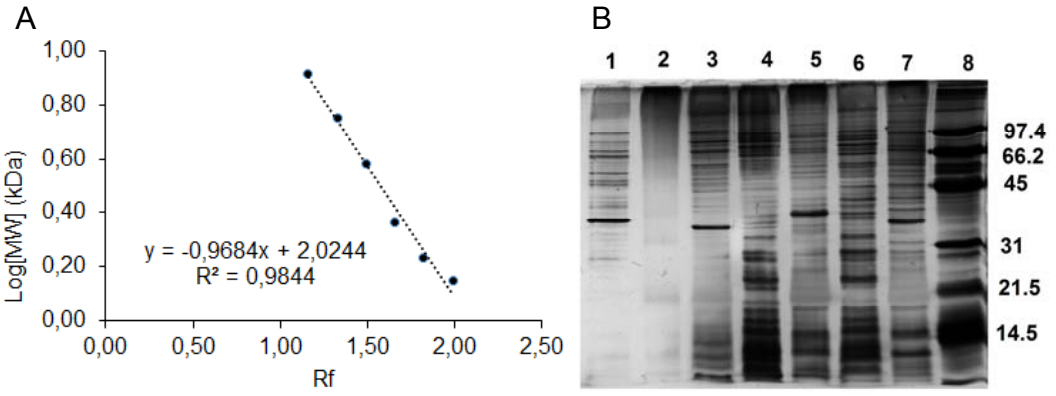


Fig. 21. A) A standard curve for low range SDS – PAGE standard at 12% gel. B) Electrophoretic separation and silver staining of proteins in the SDS – PAGE. Numbers in the right side of the gel indicate for molecular masses in kDa. Lane 1: antigen *P. skyensis*; Lane 2: antigen AL122 (*V. anguillarum* O2); Lane 3: antigen *Pasteurella* – spp.; Lane 4: antigen 8546 (atypical *A. salmonicida*); Lane 5: antigen 8752 (*V. anguillarum* O1); Lane 6: antigen AL20460 (atypical *A. salmonicida*); Lane 7: antigen 8657 (*V. ordalii*).; Lane 8: low range SDS - PAGE standards (BIO-RAD, 2015).

3.3 Western blot

Antiserum from fish immunized with formalin inactivated WB from strain 8546 (atypical *A. salmonicida*) reacted specifically with several components of a homologous antigen, having apparent MWs of 10, 13, 20, 22, 28, 50, 55 and 67 kDa (fig. 22A, Lane 2). Similarly, the same antiserum recognized similar antigenic components of a heterogeneous antigen from strain AL20460 (atypical *A. salmonicida*) (fig. 22A, Lane 1), but failed to recognize the 50-kDa protein, which was observed on the SDS-PAGE in strain AL20460 (atypical *A. salmonicida*) (fig. 21B, lane 6).

Likewise, antiserum to AL20460 (atypical *A. salmonicida*) reacted with homologous as well as heterogeneous antigens in similar pattern as the antiserum to 8546 (atypical *A. salmonicida*) (fig. 22B, Lanes 1 & 2). However, the antibodies to strain AL20460 (atypical *A. salmonicida*) reacted strongly with a 50-kDa protein from its own antigen and cross-reacted less strongly with another ca. 50 kDa protein component from strain 8546 (atypical *A. salmonicida*). Both antisera to strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) had antibodies that cross-reacted weakly with antigenic components from other unrelated strains (fig. 22A, lanes 4, 5 & 6).

Specific antibodies raised against strain AL122 (*V. anguillarum* O2) cross-reacted strongly with ca. 14-kDa antigenic component of strain 8752 (*V. anguillarum* O1) (fig. 22C, lane 4) and 8657 (*V. ordalii*) (fig. 22C, lane 6), but reacted very weakly with similar 14 kDa protein of a homologous antigen (fig. 22C, lane 7).

The antiserum raised against *Pasteurella* – spp. contained specific antibodies directed to ca. 100 and 20 kDa proteins from a homogenous antigen (fig. 22D, lane 5); the same antiserum contained antibodies cross reactive to ca. 73 kDa, 100, 37 and 20 kDa proteins from a heterogeneous antigen of *P. skyensis* (fig. 22D, lane 3). In addition, some weak cross – reactions were observed with antigenic components from strains AL20460 (atypical *A. salmonicida*) and 8546 (atypical *A. salmonicida*) (fig. 22D, lanes 1 & 2).

Western blot analysis using antisera to strain 8657 (*V. ordalii*) showed strong specific reactivity with ca. 45 kDa protein, but weakly with ca. 14 kDa protein of a homogeneous antigen (fig. 22E, lane 6). No cross reactivity was observed with antigens from other strains, but the weak 14 kDa protein from strain 8657 made strong cross-reactivity with antibodies to strain AL122 (*V. anguillarum* O2).

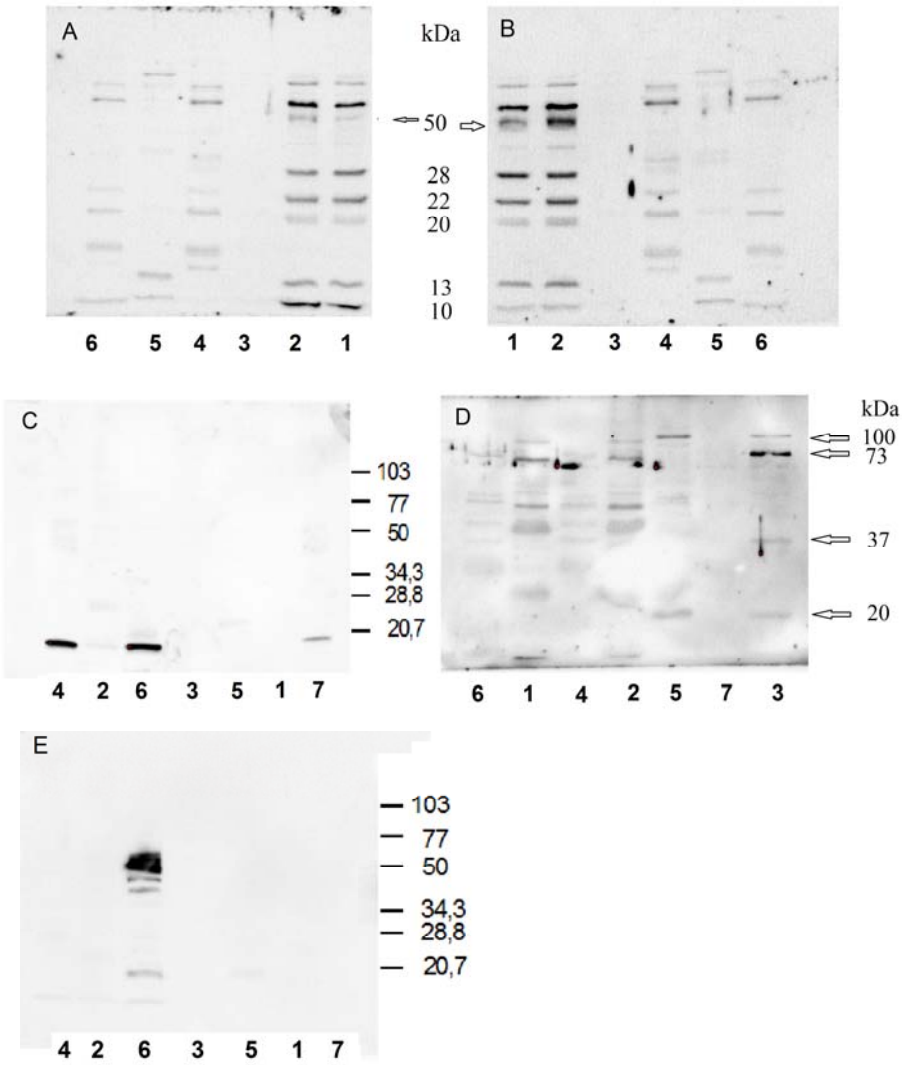


Fig. 22. Serum antibody specificity and cross – reactivity analysed by western blot. Antisera to different formalin inactivated WB antigens were used; the black blots on the pictures indicate for serum antibody reactivity with heterogeneous or homogeneous sonicated WB antigens of the different bacterial strains. A) Antiserum to 8546 (atypical *A. salmonicida*), B) Antiserum to AL20460 (atypical *A. salmonicida*), C) Antiserum to AL122 (*V. anguillarum* O2), D) Antiserum to *Pasteurella* spp., and E) Antiserum to 8657 (*V. ordalii*). Lane 1: antigen from AL20460 (atypical *A. salmonicida*); Lane 2: antigen from 8546 (atypical *A. salmonicida*); Lane 3: antigen from *P. skyensis*; Lane 4: antigen from 8752 (*V. anguillarum* O1); Lane 5: antigen from *Pasteurella* spp.; Lane 6: antigen from 8657 (*V. ordalii*); Lane 7: antigen from AL122 (*V. anguillarum* O2).

3.4 Purified lumpfish IgM

The concentration of purified lumpfish IgM was estimated at 0.5 mg/ml based on the linear formula of the standard curve of BSA shown in fig. 23. The concentration of the sample was equivalent to 0.471 ODs (595 nm).

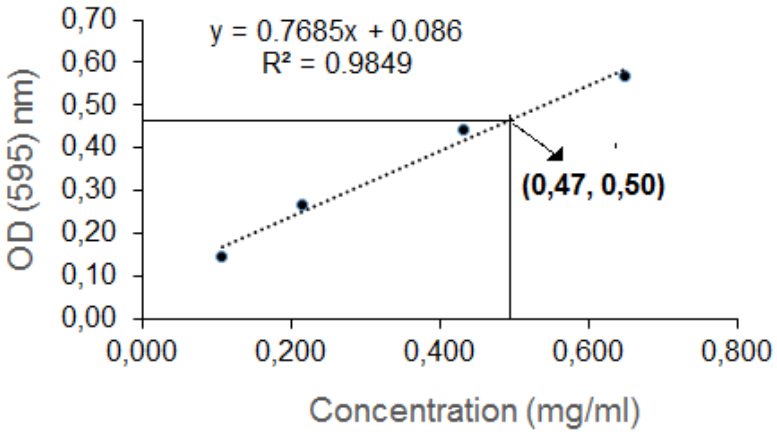


Fig. 23. A standard curve for BSA concentration (mg/ml).

3.5 Total serum IgM

Total IgM (mg/ml) in the lumpfish serum two weeks post immunization was determined by single radial immuno-diffusion assay (SRID). The rabbit anti-lumpfish Ab bound to IgM in lumpfish serum, forming rounded opaque regions (immune complexes), which were distinguished by staining with Comassie blue (fig. 24). The increment in the size of the circles ceased after all the IgM in test serum was bound to the rabbit anti-lumpfish antibody in the gel. The amount of IgM present in the antiserum was directly proportional to the diameter of the circle.

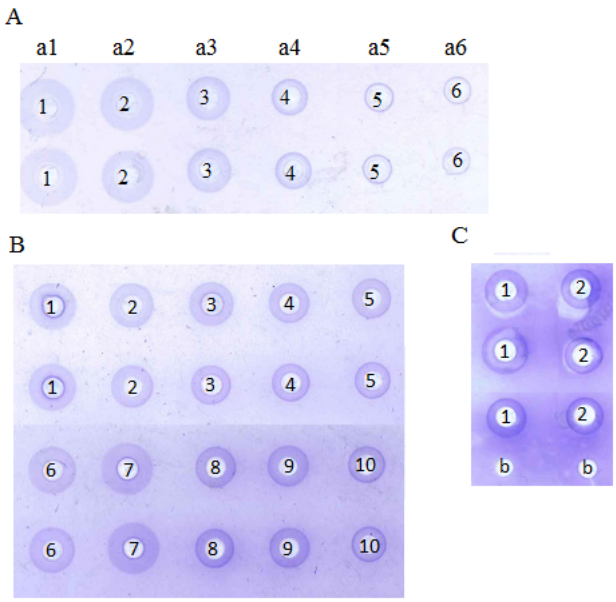


Fig. 24. Quantification of lumpfish IgM by SRID. A) Purified lumpfish IgM at different concentrations. B) IgM in antiserum from immunized groups with unknown IgM concentration. C) IgM in serum from non – immunized groups. Serum was diluted 1/20 in PBS (Lonza). Numbers inside the rings indicate for samples in duplicates or triplicates. Symbols from a1 to a6 indicate for IgM concentrations. a1, 160 ng/µl; a2, 80 ng/µl; a3, 40 ng/ µl; a4, 20 ng/ µl; a5, 10 ng/µl; a6, 5 ng/µl; b, blank (PBS without IgM).

The total IgM in sera was quantified based on a linear formula of a standard curve for the purified lumpfish IgM (fig. 25A). Mean total IgM for the different immunized groups ranged from 1.02 mg/ml to 0.41 mg/ml (fig. 25B, table 9). Antisera from fish immunized with AL122 (*V. anguillarum* O1) contained the highest IgM followed by antisera from fish immunized with strain 8546 (atypical *A. salmonicida*), AL20460 (atypical *A. salmonicida*), 8657 (*V. ordalii*) and *Pasteurella* spp. (table 7); however, the total IgM in sera from immunized groups was not different from the total IgM in sera from non – immunized group ($p>0.05$). Moreover, total IgM in sera from another non-immunized fish group was also determined by SRID. This group was comprised of smaller and younger fish individuals than the other non – immunized group (800 gm, 21 cm), which was used for comparison with immunized groups. The total serum IgM in the smaller and younger fish was estimated at 0.14 ± 0.10 mg/ml (fig. 25C).

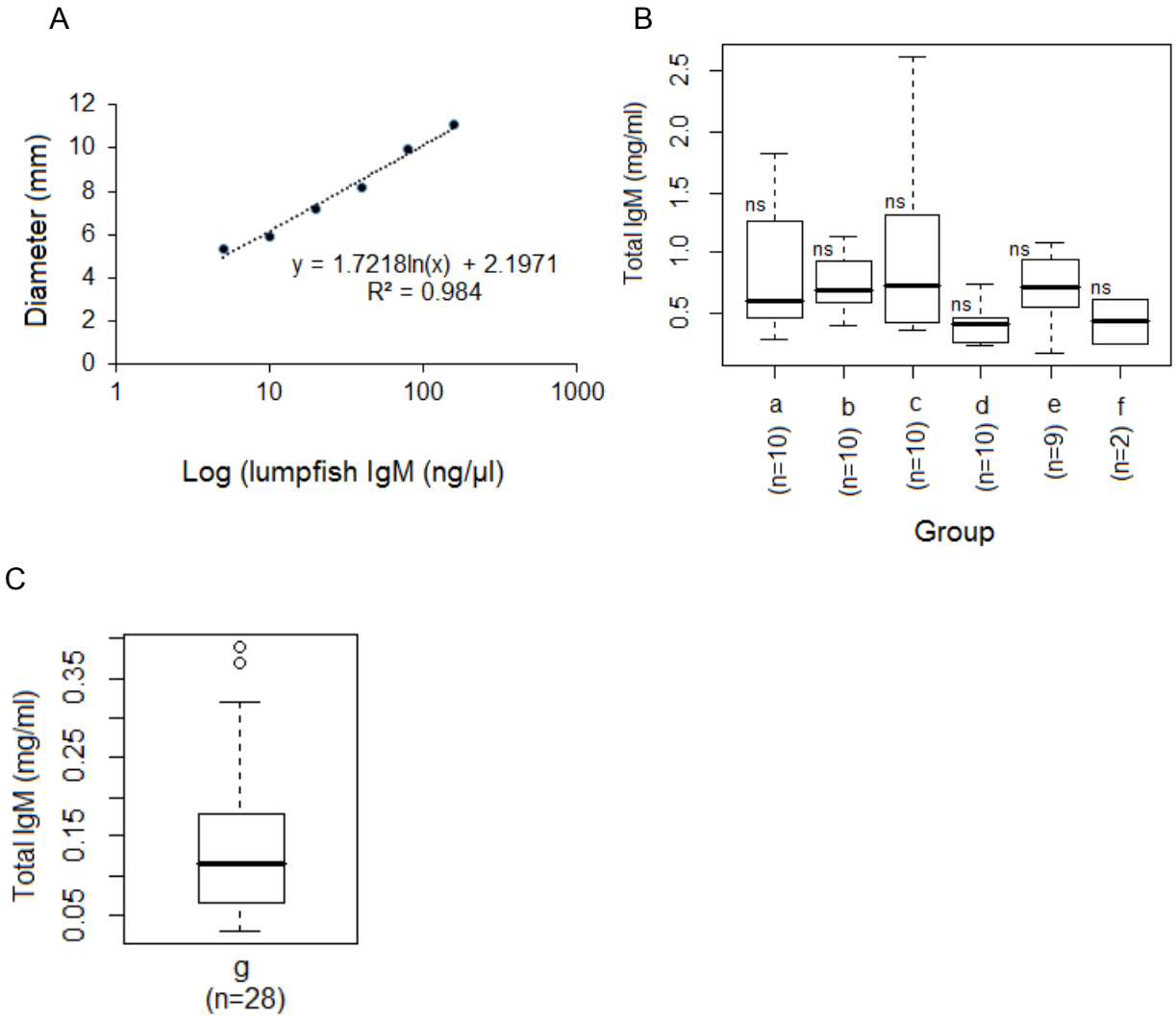


Fig. 25. A) A standard curve for purified lumpfish IgM (ng/µl). B) Total serum IgM (mg/ml) in immunized and non-immunized groups (800 gm, 21 cm) determined by SRID. C) Total serum IgM (mg/ml) in the smaller and younger non – immunized fish. The vertical lines indicate for standard deviations. a, 8546 (atypical *A. salmonicida*); b, AL20460 (atypical *A. salmonicida*); c, AL122 (*V. anguillarum* O2); d, *Pasteurella* spp.; e, (*v. ordalii*); f, non – immunized fish (800 gm, 21 cm); g, non-immunized fish.

Table 7. Total serum IgM (mg/ml) in the lumpfish two weeks post immunization.

Group	**N**	**Mean total IgM ± SD(mg/ml)**
8546 (atypical *A. salmonicida*)	10	0.80 ± 0.50
AL20460 (atypical *A. salmonicida*)	10	0.74 ± 0.23
AL122 (*V. anguillarum* O2)	10	1.02 ± 0.76
Pasteurella – spp.	10	0.41 ± 0.15
8657 (*V. ordalii*)	9	0.67 ± 0.31
Non - immunized	2	0.62 ± 0.25

4. DISCUSSION

The aim of this study is to investigate the presence of specific antibody response in sera of the lumpfish after immunization, vaccination and challenge with different bacterial pathogens, to test the occurrence of cross-reactive antibodies between different bacterial strains and to estimate total serum IgM in the lumpfish.

SDS-PAGE analysis of proteins: Protein profiles of the different sonicated WB antigens were carefully examined by 12% SDS – PAGE and silver staining. Strain AL122 (*V. anguillarum* O2) showed no protein bands on the SDS PAGE. The sample was probably destroyed during preparation, hence it was not suitable for analysis of protein profiles by SDS-PAGE. However, all the other strains displayed very heterogeneous sizes of LMW and HMW components across the SDS-PAGE between 97 and 14 kDa (fig. 21B). Several noticeable HMW bands (>45 kDa), closely spaced to each other, were also visible on the SDS – PAGE for most of the samples.

Strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) had similar protein profiles with some differences (fig. 21B, lanes 4 & 6). Both strains produced several major protein bands with apparent MWs of 32, 30, 23, 20 and 14 kDa, and also had similar band patterns greater than ca. 66 kDa. Unlike strain AL20460 (atypical *A. salmonicida*), strain 8546 (atypical *A. salmonicida*) lacked a few visible protein bands of ca. 37, 40, and 45 kDa and a weak band of ca. 50 kDa. A protein with a MW of 20 kDa protein band in strains that belong to atypical *A. salmonicida* subsp. *achromogenes* has been described as caseinolytic and gelatinolytic extracellular metalloprotease, an essential virulent factor (Warr, 1997). However, the proteolytic activity of the 20 kDa in strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) was not verified by this study. Thus, further studies are needed to confirm the presence of extracellular metalloprotease characterized as 20 kDa protein in the atypical *A. salmonicida* strains. *A. salmonicida* subsp*. achromogenes protease* 1 (AsaP1), like many

of the toxic proteases, is dependent on Zn^{+2} to perform its toxic effect against the host. AsaP1 can be toxic to several fish species including Arctic charr, Atlantic salmon, and Atlantic cod (Magnadóttir, 1998). It has been suggested that antibodies directed against AsaP1 can induce protection in fish against atypical furunculosis caused by atypical *A. salmonicida* subsp. *achromogenes* (Kunihiko et al., 1984). Another virulent factor in *A. salmonicida* is the A – layer (Sanchez et al., 1989), a 49 kDa (approx. 50 kDa) component of the OM (Hsu and Du Pasquier, 1984). SDS – PAGE analysis on strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) revealed the presence of a faint 50 kDa protein in strain AL20460 (atypical *A. salmonicida*), which could be an A - layer, but absent in strain 8546 (atypical *A. salmonicida*). It was suggested that the genes that encode the synthesis of the A – layer in strain 8546 may not be expressed. According to some authors, the gene that expresses the A – layer in some isolates of atypical *A. salmonicida* subsp. *achromogenes* may undergo irreversible changes, distorting the functionality of the A – layer (Gulla et al., 2015, Merino et al., 2015). However, these isolates remained virulent in spite of losing functional A – layer (Gulla et al., 2015). The A – layer is a vital component of the OM; it prevents the bacterium from serum effect (Mestecky et al., 1975), proteolytic action (Anderson et al., 1974) and phagocytosis (Weinheimer et al., 1971). Moreover, it can increase binding to some, Ig and many extracellular proteins (Weinheimer et al., 1971).

Strain 8752 (*V. anguillarum* O1) produced a major protein of ca. 38 kDa, a diffused (bulged) band of 21 kDa, and a broad band of ca. 14 kDa (fig. 21B, lane 5). It has been described that different serotypes of *V. anguillarum* have a major outer membrane protein (MOMP) with a molecular weight ranging between 35 and 42 kDa (Simon et al., 1996). *V. anguillarum* serotype O2 isolates may possess MOMPs in the range of 35 – 38 kDa (Santos et al., 1995). It has been proposed that this MOMP in *V. anguillarum* is a porin (Suzuki et al., 1994). Porins might play an important role in environmental adaptation by allowing selective movement of molecules into the cell membrane through their hydrophilic channels while the OM forms a barrier to protect the cell from harmful substances (Wang et al., 2003). Environmental parameters such as osmotic concentration, temperature, nutrient limitation and phosphate can affect the synthesis of

porins (Wang et al., 2003). Moreover, porins may play a crucial role in bile resistance and biofilm formation, which are critical to the survival of the bacterium inside the fish or in the marine environment (Wang et al., 2003).

Likewise, strain 8657 (*V. ordalii*) had a MOMP with approximate MW of 36 kDa and a LMW protein of ca. 14 kDa (fig. 21B, lane 7). The 14 kDa LMW protein was probably associated with LMW LPS, but it was not verified in this study. The results from strain 8657 (*V. ordalii*) seem to agree with the results from SDS-PAGE analysis on OMP of *V. ordalii* by Akayli et al., (2010). The MOMP, together with the LPS, in *V. ordalii* is considered as a major epitope (Chart and Trust, 1984). Strains 8657 (*V. ordalii*) and 8752 (*V. anguillarum* O1) seemed to share similar OM structures below ca. 31 kDa protein band.

SDS – PAGE analysis on sonicated WB antigens of *P. skyensis* and *Pasteurella* - ssp. revealed the presence of major proteins weighing ca. 37 and 35 kDa, respectively (fig. 21B, lanes 1 & 3). The nature of these bands is not known as the bacteria are not well studied. The *P. skyensis* and *Pasteurella* spp. belong to *Pasteurellaceae*, a family which encompasses a diverse group of bacteria (Challacombe and Inzana, 2008) whose taxonomic positions have been unresolved (Korczak et al., 2004).

Antibody response to immunization. The lumpfish were immunized three times; the antiserum was collected two weeks post immunization. The ELISA results showed that immunization of lumpfish against the different formalin inactivated WB antigens induced elevated levels of specific antibodies compared to the non-immunized control group (fig. 10 & 11), revealing adaptive humoral immune response in the lumpfish. The highest antibody response was attained by immunization to *Pasteurella* spp., followed by strain AL122 (*V. anguillarum*), AL20460 (atypical *A. salmonicida*), 8546 (atypical *A. salmonicida*), and *V. ordalii*). However, the level of antibody response to strain 8652 (*V. ordalii*) was not significantly different in comparison to the non-immunized control group (fig. 11B). The amount of specific antibodies varied within individuals and between groups. A number of factors can affect antibody response in fish; some of these factors

include antigen dose, type of antigen, adjuvants, route of administration, age, size, genetic background, and booster administration (Morrison and Nowak, 2002).

Repeated immunization of the lumpfish could have contributed to the production of specific antibodies with the second or third immunization producing specific antibodies of superior quality and quantity (Morrison and Nowak, 2002). In higher vertebrates, the high affinity binding of specific antibodies is attributed to affinity maturation of the B – cells, a mechanism which is not fully understood in many fish species. However, increased and improved secondary antibody response with high specificity to antigens has been demonstrated in fish (Fletcher and Secombes, 2010). Nevertheless, this study may suggest that the demonstration of specific antibodies by ELISA may give an important glimpse to further delve into the lumpfish's specific humoral immune response, particularly with respect to protective immunity that involves immunization or vaccination and challenge against the different bacterial pathogens. However, it should be noted that the presence of specific antibodies in fish should not be always correlated with protection against bacterial pathogens (Morrison and Nowak, 2002).

Antibody specificity and cross-reactivity: Western blot analysis revealed that antisera to strains 8546 (atypical *A. salmonicida*) and AL20460 (atypical *A. salmonicida*) contained antibodies reactive to several antigenic components commonly shared by both strains (fig. 22A & 22B; lanes 1 & 2). The specificities of the antibodies to both atypical *A. salmonicida* strains were directed to ca. 10, 13, 20, 22, 28, 50, and 55 kDa proteins. The cross – reactivity between the two strains were also revealed by ELISA. Unlike in strain AL20460 (atypical *A. salmonicida*), a band of ca. 50 kDa A-layer protein was not observed in strain 8546 (atypical *A. salmonicida*) by SDS-PAGE analysis on sonicated WB antigens (fig. 21B, lanes 6 & 4). Moreover, the 50 kDa A-layer protein in strain AL20460 (atypical *A. salmonicida*) was not detected when using antiserum to strain 8546 (atypical *A. salmonicida*) by the western blot. According to the results from ELISA, immunization with strain AL20460 (atypical *A. salmonicida*) resulted in relatively higher specific antibody response than immunization with strain 8546 (atypical *A. salmonicida*) ($p<0.01$) (fig 11). Thus, the difference in the level of specific antibodies between these

two immunized groups could be attributed to the 50 kDa A – layer protein.

The 50 kDa protein was already described as an A – layer protein and the LMW protein bands of less than 20 kDa were perhaps linked to LPS. In some other studies, the A – layer was shown to elicit good specific humoral immune response in Atlantic salmon (Lund et al., 1991) and rainbow trout (Thuvander et al., 1993). The A – layer in *A. salmonicida* is bound to the LPS (Belland, 1985) and covers almost the whole bacterium (Dooley et al., 1989), nonetheless some LPS molecules are found uncovered (Phipps and Kay, 1988). Both of these OM structures are important to protect the bacterium from inactivation by serum components (Munn et al., 1982), but the A – layer is a more vital armour against opsonophagocytosis (Merino et al., 1994). It has been reported that the LPS and A-layer proteins of the atypical *A. salmonicida* can induce specific antibodies in salmon (Evenberg et al., 1985), rainbow trout (Hastings and Ellis, 1990), spotted wolfish (*Anarhichas minor*) and halibut (*Hippoglossus hippoglossus*) (Ingilæ et al., 2000).

Immunoblotting studies using antiserum to AL122 (*V. anguillarum* O2) displayed strong cross-reactivity with strains 8752 (*V. anguillarum* O1) (fig. 22C, lane 4) directed to ca. 14 kDa protein. Moreover, elevated levels of antibodies were also demonstrated by ELISA due to the cross reactivity of the specific antibodies to strain AL122 (*V. anguillarum* O2) with sonicated WB antigens of strain 8752 (*V. anguillarum* O1) (fig. 13). The high antibody response to strain 8752 (*V. anguillarum* O1) was possibly attributed to the 14 kDa protein. However, the level of antibodies due to the cross reactivity were not significant compared to the level of antibodies in serum from the non-immunized control group ($p>0.05$). This result was unexpected as one of the sera from the non-immunized control group made elevated levels of antibodies against sonicated WB antigen of strain 8752 (*V. anguillarum* O1), which was used as a coating antigen. It was proposed that the serum from the non-immunized control group might possibly have contained some natural antibodies reacting with some components of strain 8752 (*V. anguillarum* O1). Natural antibodies may arise due to contact of the fish with bacterial pathogens in the marine or culture environment (Espelid et al., 1991).

Similarly, specific antibodies to AL122 (*V. anguillarum* O2) cross reacted strongly with a 14 kDa antigenic component of strain 8657 (*V. ordalii*) (fig. 22C, lane 6). Cross reactivity test was not performed by ELISA between the *V. anguillarum* O2 and *V. ordalii* strains. A number of authors have demonstrated extensive cross reactions with LPS using polyclonal antisera against *V. ordalii* and *V. anguillarum* O2 (Chart and Trust, 1984, Mutharia et al., 1993). However, this study reported no cross-reactivity with sonicated WB antigens of strain AL122 (*V. anguillarum* O2), also with strain 8752 (*V. anguillarum* O1) when using antiserum to 8657 (*V. ordalii*) (fig. 22E, lanes 4 & 7).

To the contrary, antibodies to AL122 (*V. anguillarum* O2) reacted very weakly with a homogeneous antigen directed to ca. 14 kDa protein (fig. 22C, lane 7) compared to the other heterogeneous antigens. No OMP bands were also observed on this strain by SDS-PAGE using sonicated WB antigens. Strain AL122 (*V. anguillarum* O2) was treated with formalin because it was supplied as vaccine antigen by PHARMAQ AS.

Western blot analysis using antiserum to strain 8657 (*V. ordalii*) revealed the presence of highly specific antibodies directed mainly to ca. 45 kDa component of a homogeneous antigen (fig. 22E, lane 6). Some antibodies were also weakly reactive with ca. 14 kDa protein of the homogeneous antigen.

It appears that all the *Vibrio* strains used in this study seemed to possess a common 14 kDa protein. This LMW 14 kDa protein was perhaps associated with LMW LPS, but not verified by this study. The LPS has been considered as significant antigenic determinant of *V. ordalii* and *V. anguillarum* (Chart and Trust, 1984); it is usually included during vaccine formulation against vibriosis (Colquhoun and Lillehaug, 2014). However, the LPS from *V. ordalii* and *V. anguillarum* O2 have different antigenic properties (Mutharia et al., 1993). Vaccines containing bacterins of *V. anguillarum* O1 and *V. ordalii* may provoke poor protection against vibriosis caused by *V. anguillarum* O2 (Toranzo et al., 1996).

The antibodies raised against *Pasteurella* spp. reacted specifically with antigenic components of a homologous antigen forming faint bands of ca. 100 kDa and 20 kDa,

respectively (fig. 22D; lane 5). Likewise, the same antisera cross-reacted with a heterogeneous antigen prepared from *P. skyensis* (fig 22D; lane 3). Both the homologous and heterogeneous antigens shared similar OM proteins of 100 and 20 kDa. Conversely, there was a strong and a faint cross reactivity at only ca. 73 kDa and 37 kDa against the heterogeneous antigen, respectively. ELISA was not performed for cross – reactivity test between *Pasteurella* spp. and *P. skyensis*. The OM proteins of both strains that were detected by the western blot and SDS – PAGE were not fully understood. However, this study revealed that the specific humoral immunity of the lumpfish were capable of responding to formalin inactivated whole cells of *Pasteurella* spp., producing significant amount of specific antibodies as determined by ELISA and western blot. Western blot analysis showed that antigen – antibody reactivity was stronger with *P. skyensis* than with the homologous antigen. Therefore, *Pasteurella* spp. from lumpfish induced antibodies to *P. skyensis*. The significance of this regarding bacterial vaccine antigens need to be further elucidated.

Furthermore, the extensive weak cross reactions between antibodies to *Pasteurella* spp. and the atypical *A. salmonicida* strains remained unclear. Sometimes, there is a possibility for antibody cross-reactivity between unrelated antigens (Murphy, 2012). In one study (Hoel et al., 1998), for example, it was shown that epitopes from *V. salmonicida* and *A. salmonicida* were cross-reactive and had protective role against typical furunculosis, vibriosis and cold water vibriosis in Atlantic salmon. In the case of the *Pasteurella* spp., the fact that the unresolved taxonomic position of the *Pasteurella* spp. may complicate our understanding of its role in the specific humoral immune response of the lumpfish.

Antibody response to challenge: Antibody levels in the small lumpfish from the non-vaccinated and vaccinated challenged groups were quantified by ELISA; in general, it was found that the specific humoral immune response in all non-vaccinated challenged groups was poor, evaluated by the presence of almost negligible specific antibody levels. The specific immune response of the lumpfish was perhaps suppressed due to challenge with the different bacteria, resulting in a low specific antibody response. For example,

Magnadóttir et al. (1995) reported poor specific antibody response in Atlantic salmon that had been chronically infected with atypical *A. salmonicida* subsp. *achromogenes.* Another explanation for the low antibody levels after exposure to challenge might be due to either formation of antigen – antibody complex, or the utilization of the complexes by opsonisation, depleting free antibodies from plasma (Villumsen et al., 2012). The mortality data were not included in the present study, and correlation between mortality and specific antibody response would possibly justify the occurrence of protection in the lumpfish against the different bacterial pathogens.

No significant antibody levels, compared to the non-challenged control group, were found in sera from fish challenged with strain 8546 (atypical *A. salmonicida*) by any of the challenge exposures (bath, i.p. or cohabitation) ($p>0.05$) (fig. 14). However, relatively higher antibody levels were detected in sera from fish challenged by cohabitation with strain AL20460 (atypical *A. salmonicida*) compared to the non-challenged control group ($p<0.01$) (fig. 15).

Higher antibody levels were found in sera from fish challenged by cohabitation or i.p. against strain 8752 (*V. anguillarum* O1) (fig. 16) compared to the non-challenged control group ($p<0.001$), but only two fish survived from i.p. challenged group. The bath challenge against strain 8752 (*V. anguillarum* O1) did not induce significant amount of specific antibodies ($p>0.05$).

Challenge with the *Pasteurella* spp. by cohabitation in 1 to 1 ratio to i.p. at different bacterial concentrations induced high antibody levels relative to the non-challenged control group ($p>0.001$). Low antibody response was found in sera from fish challenged with the highest challenge dose (1/20). The fish challenged with lowest dilution (1/20) had high mortality (Anita R., personal communication) and fish used for serum sample might be infected or antibodies were bound in immune complexes.

On the other hand, the amount of specific antibody response in vaccinated, challenged groups (i.p.) was much higher than the non-vaccinated challenged (i.p.) and

non-challenged control groups (PBS) (fig. 14). However, western blot was not performed for these groups to confirm antibody specificity or cross-reactivity. The presence of specific antibodies - one of the protective factors – may give an indication of some degree of protection in the lumpfish against vibriosis, atypical furunculosis and pasteurellosis. Generally, vaccines against vibriosis (Colquhoun and Lillehaug, 2014) and pasteurellosis, caused by *Photobacterium damselae* subsp. *Piscicida* have been effective for many marine fish species. However, there is still challenge for finding protective vaccines for infections caused by atypical *A. salmonicida* (Midtlyng, 2014). Nevertheless, the protective role of the specific antibodies due to vaccination of the lumpfish should be investigated by comparisons with the relative percent of survival (RPS) based on mortality data of vaccinated and non-vaccinated challenge groups. The elevated antibody levels in the vaccinated challenged groups was perhaps due to high-affinity antibodies induced by the vaccines, though this requires further investigation. In one study, it was suggested that vaccines against *V. anguillarum* resulted in significant specific antibody response in zebra fish (Zhang et al., 2013). In another study, vaccination of rainbow trout enhanced IgM productivity, resulting in increased specific antibody titre and antibody affinity to pathogens (Costa et al., 2012). Furthermore, the presence of high specific antibodies in all vaccinated groups could be due to the fact that i.p. vaccination might cause marked antibody response (Harrell et al., 1976) that may last for several weeks (THUVANDER et al., 1987).

In groups challenged against 8546 (atypical *A. salmonicida*), lumpfish that were treated with trivalent vaccines produced slightly higher specific antibodies than those fish treated with monovalent vaccines ($p<0.05$), but antibody responses did not differ between sera from fish that were treated with trivalent and AMM3 vaccines ($p>0.05$). On the other hand, the specific antibody levels within groups that were treated with the different vaccines were similar ($p>0.05$) 31 days post challenge against strains AL20460 (atypical *A. salmonicida*) and 8752 (*V. anguillarum* O1). The AMM3 and trivalent vaccines against atypical furunculosis induced better antibody response 43 days post challenge with strain 8546 (atypical *A. salmonicida*) than 31 days post challenge with strain AL20460 (atypical *A. salmonicida*) ($p<0.05$). This result was in contrary with the ELISA results from the

immunization, in which higher antibodies were obtained with immunization to strain AL20460 (atypical *A. salmonicida*) than with strain 8546 (atypical *A. salmonicida*). The difference in the effectiveness of the vaccines in terms of antibody response might be linked to antigenic composition of the vaccines, though the exact composition of the vaccines is kept confidential by the commercial vaccine producers, also during vaccine trial. It has been reported in some studies that inclusion of multiple WB antigens in vaccines may have inhibitive (Nikoskelainen et al., 2007) or enhancive (Hoel et al., 1997) effects on the specific humoral immune response of fish. Nikoskelainen et al. (2007) proposed that the specific humoral immune response of Atlantic salmon (*Salmo salar* L.) to *A. salmonicida* was more enhanced by oil-adjuvanted polyvalent vaccine that incorporated *V. salmonicida, V. anguillarum* (O1 and O2) and *A. salmonicida* than a monovalent vaccine containing only *A. salmonicida*; however, immunological cross-reaction between *V. salmonicida* and *A. salmonicida* was the main factor for the enhancement of the humoral immune response in the Atlantic salmon (Nikoskelainen et al., 2007). Western blot analysis showed cross-reactions between atypical *A. salmonicida* and *V. anguillarum* when using antisera to either strains of atypical *A. salmonicida.* Likewise, there were cross reactions between *Pasteurella* spp. and atypical *A. salmonicida*, but when using antiserum to *Pasteurella* spp. (fig. 22). No other works have reported on cross-reactions between these bacteria. Thus, other studies are required to investigate the finding, and their protective role in vaccines for the lumpfish should be investigated.

Total serum IgM: Total serum IgM (mg/ml) in lumpfish was determined by SRID; mean total serum IgM (mg/ml) for all groups ranged from 1.02 to 0.41 mg/ml (fig. 19B & Table 7). No significant differences in total serum IgM (mg/ml) were observed between immunized and non-immunized groups ($p>0.05$). Individual variation in total serum IgM was observed. Moreover, the total serum IgM in the small and young fish was estimated at 0.14 ± 0.10 mg/ml. Total serum IgM (mg/ml) in the lumpfish was relatively low compared to other fish species but the results obtained for the lumpfish fell within the range of the total serum IgM (mg/ml) for other fish species as reported by Israelson et al., (1991). Total serum IgM varies among fish species (Israelson et al., 1991); several factors may affect

the level of serum IgM in fish including age or size, environmental conditions (Scapigliati et al., 1999), nutrition (immunostimulators) (Cuesta et al., 2004), etc. Based on the results by Israelson et a., (1991), Atlantic cod (*Gadus morhua*) contains 7 – 8 times more IgM than the lumpfish but, quantitatively, the total serum IgM between Atlantic salmon (*Salmo salar* L.) and the lumpfish is nearly similar. The variation in the amount of serum IgM between fish species may indicate for differences in their specific humoral immune responses (Magnadóttir, 1998). For example, salmon is capable of generating highly specific antibodies of less diversity, while cod produces high amount of natural antibodies of low specificity (Magnadóttir, 1998).

5. CONCLUSION

The most important conclusion of this project is that the lumpfish's adaptive humoral immune responses, like many other aquaculture fish species, is capable of responding to i.p. immunization or vaccination with formalin inactivated WB antigens and challenge with different bacteria by producing high levels of specific antibodies. This indicate that the lumpfish can be protected from the most common bacterial pathogens. However, the protective role of the specific antibodies in the lumpfish against the different bacteria should be thoroughly investigated by further studies with respect to protective immunity, involving challenge, mortality and vaccination trials. Moreover, it can be concluded that the lumpfish have normal IgM levels comparable to what is found in Atlantic salmon, but lower than that of Atlantic do.

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7. APPENDIX A. List of chemicals and solutions

No.	Ingredients	Quantity	Company
1.	**Agarose Gel**		
	• Agarose 1%	0.335 g	Lanzo Group Ltd.
	• PBS (pH =7.2)	33.35 ml	Lanzo Group Ltd.
	• PEG-6000	0. 67 g	Sigma Aldrich, Co.
2.	**PBS (pH = 7.2)**		
	• $Na_2HPO_4x2H_2O$	0.72 g	Sigma Aldrich, Co.
	• KH_2PO_4	0.27 g	Sigma Aldrich, Co.
	• NaCl	8.5 g	Sigma Aldrich, Co.
	• Deionized H_2O	1000 ml	Milli Q
3.	**PBS-T (100ml)**		
	• PBS (pH=7.3)	100 ml	
	• 0.05% Tween 20	50 µl	Sigma Aldrich, Co.
4.	**Blocking solution**		
	• Skim milk powder	3 g	Sigma Aldrich, Co.
	• PBS-T	100 ml	
5.	**0.1M Citric acid**		
	• Citric acid	21 g	Sigma Aldrich, Co.
	• Deionized H_2O	1000 ml	MilliQ
6.	**0.2 M $Na_2HPO_4x2H_2O$**		
	• $Na_2HPO_4x2H_2O$	35.3 g	Sigma Aldrich, Co.
	• Deionized H_2O	1000 ml	MilliQ
7.	**Phosphate citrate buffer, pH = 5.0**		
	• 0.1 M citric acid	24.3 ml	
	• 0.2M $Na_2HPO_4x2H_2O$	25.7 ml	
	• Deionized H_2O	50 ml	MilliQ

List of chemicals and solutions (Continues)

8. Peroxidase substrate solution

• O – phenyleneidamine (OPD, P-4664)	15 mg (1 tablet)	Sigma Aldrich, CO.
• Phosphate citrate buffer	37.5 ml	
• 30% H_2O_2	15 µl	Sigma Aldrich, Co.

9. PBS with 5M EDTA, pH = 7.3

• PBS, pH 0 7.3	100 ml	
• EDTA	0.2 g	Merck

10. TSB with 2% NaCl

• TSB	30 g	Bacto™
• NaCl	15 g	Sigma Aldrich, Co.
• Deionized H_2O	1000 ml	MilliQ

11. 2.5N H_2SO_4

• 96% H_2SO_4	6.94 ml	Merck
• Deionized H_2O	93.06 ml	MilliQ

12. Coomassie staining solution (1000 ml)

• 98.6% Methanol	400 ml	Sigma Aldrich, Co.
• 98.6% Acetic acid	100 ml	Sigma Aldrich, Co.
• Commassie Brilliant Blue G-250	500 mg	Merck
• Deionized H_2O	500 ml	MilliQ

13. Commassie destaining (1500 ml)

• 98.6% Methanol	400 ml	Sigma Aldrich, Co.
• 98.6% Acetic acid	100 ml	Sigma Aldrich, Co.
• Deionized H_2O	500 ml	MilliQ
• Commassie staining solution	500 ml	

List of chemicals and solutions (Continues)

14. Resolving gel (12%)		
• Deionized H_2O	4.4 ml	MilliQ
• 30% Degassed Acrylamide/Bis	3 ml	Sigma Aldrich, Co.
• 1.5 M Tris-HCl, pH 8.8 (Gel buffer)	2.5 ml	
• 10 % w/v SDS	100 µl	
• 10 % APS	50 µl	
• TEMED	5 µl	Sigma Aldrich, Co.
15. 10 % SDS		
• SDS	10 g	Sigma Aldrich
• Deionized H_2O	90 ml	MilliQ
• Total volume brought to 100 ml with deionized H_2O		
16. 10% APS		
• Ammonium sulfate	100 mg	Sigma Aldrich, Co.
• Deionized H_2O	1 ml	MilliQ
17. 1.5 M Tris-HCl, pH 8.8 (150 ml)		
• Tris base	27.23 g	Sigma Aldrich, Co.
• Deionized H_2O	80 ml	MilliQ
• pH adjusted to 8.8 by adding 6N HCl and total volume brought to 150 ml by adding deionized H_2O.		
18. Stacking gel (4%)		
• Deionized H_2O	6.4 ml	MilliQ
• 30% Degassed acrylamide/Bis	1 ml	Sigma Aldrich, Co.
• 0.5 M Tris-HCl, pH 6.8	2.5 ml	
• 10% w/v SDS	100 µl	
• 10 % APS	50 µl	
• TEMED	10 µl	Sigma Aldrich, Co.

List of chemicals and solutions (Continues)

19. 0.5 M Tris-HCl pH 6.8

• Tris base	6 g	Sigma Aldrich, Co.
• Deionized H_2O	60 ml	MilliQ

• pH adjusted to 6.8 by adding 6N HCl and total volume brought to 100 ml by adding deionized H_2O.

20. 10xTGS buffer (1000 ml)

• Tris base	30.3 g	Sigma Aldrich, Co.
• Glycine	144 g	Sigma Aldrich, Co.
• SDS	10 g	Sigma Aldrich, Co.
• Deionized H_2O	1000 ml	MilliQ

21. 1xTGS buffer (1000 ml)

• 10xTGS buffer	100 ml	
• Deionized H_2O	900 ml	

22. Gel-loading buffer

• Sample buffer	950 µl	
• 2-mercaptoethanol	50 µl	Sigma Aldrich, Co.

23. Fixative solution

• 98.6% Methanol	100 ml	Sigma Aldrich, Co.
• 98.6% Acetic acid	20 ml	Sigma Aldrich, Co.
• Fixative enhancer concentrate	20 ml	Bio-Rad
• Deionized H_2O	20 ml	MilliQ

24. Silver staining solution

• Deionized H_2O	45 ml	MilliQ
• Silver complex solution	5 ml	Bio-Rad
• Reduction moderator solution	5 ml	Bio-Rad
• Image development reagent	5 ml	Bio-Rad
• Development accelerator solution	50 ml	Bio-Rad

List of chemicals and solutions (Continues)

25. 5 % Acetic acid (100 ml)		
• 98.6% Acetic acid	5 ml	Sigma Aldrich
• Deionized H_2O	95 ml	MilliQ
26. 10xTransfer buffer without methanol		
• Tris base	15.4 g	Sigma Aldrich, Co.
• Glycine	72 g	Sigma Aldrich, Co.
• Deionized H_2O	500 ml	MilliQ
27. 1xTransfer buffer		
• 10xTransfer buffer	100 ml	
• Deionized H_2O	700 ml	MilliQ
• 98.6% Methanol	200 ml	Sigma Aldrich, Co.
(Added just before use)		
28. 10xTBS (pH 7.5)		
• Tris base	12.11 g	Sigma Aldrich, Co.
• NaCl	146.1 g	Sigma Aldrich, Co.
• Deionized H_2O	500 ml	MilliQ
29. 1xTBS (pH 7.5)		
• 10xTBS	100 ml	
• Deionized H_2O	900 ml	MilliQ
30. TTBS		
• 1xTBS (pH7.5)	700 ml	
• 0.05% Tween-20	350 µl	Sigma Aldrich, Co.
31. TBS with 3% skimmed milk powder (100ml)		
• 1xTBS	100 ml	
• Skim milk powder	3 g	Sigma Aldrich, Co.
32. Antibody dilution buffer (100ml)		
• TTBS	100 ml	
• Skim milk powder	1 g	Sigma Aldrich, Co.

8. APPENDIX B. Silver staining protocol for protein separation on 12% SDS – PAGE.

This is the same protocol used by the Fish Immunology Group at the University of Bergen.

1. Fixation of proteins after separation by electrophoresis
    - Fix the proteins in the gel for 20 minutes in a mixture of 100 ml methanol (Sigma Aldrich), 20 ml acetic acid (Sigma Aldrich), 20 ml fixative enhancer concentrate (BIORAD) and 20 ml deionized water (MilliQ).
2. Washing
    - Wash the gel in MilliQ for 2 x 10 minutes
3. Preparation of coloring solution
    - Prepare a coloring solution by adding, in a consecutive order, 5 ml silver complex solution (solution1) (BIORAD), 5 ml reduction moderator solution (solution2) and 5 ml image development reagent (solution3) into 45 ml deionized water (MilliQ). Stir the solution.
    - Finally add 50 ml development accelerator solution (BIORAD) into the mixture. Stir the solution.
4. Staining
    - Stain the proteins in the gel with the coloring solution for ca. 45 minutes. Watch the gel continuously, and avoid overexposure. Observe for development of brown colored bands on the gel.
    - Stop the reaction by replacing the coloring solution with 5% acetic acid. The gel can stay in 5% acetic acid for 24 hours until it is transferred into deionized water.
    - Take photos of the protein bands on the gel for documentation.

9. APPENDIX C. List of tables of results

TABLE C1. Antibody response (OD_{492}) to immunization for antigen optimization by ELISA using sonicated WB cells from strain 8546 (atypical *A. salmonicida*) as antigen at different concentrations.

Plate No.	Ag (µg/ml)	Ab Dilution	Ag blocked with 3% skimmed milk						Ag not blocked					
			B	F 1	F1	F2	F2	B	F1	F1	B	F2	F2	B
1	10	50	0,20	1,23	1,20	1,13	1,12	0,26	1,30	1,27	0,30	1,16	1,14	0,29
		100	0,23	1,19	1,19	0,98	1,05	0,34	1,18	1,16	0,30	1,08	1,01	0,29
		200	0,09	1,08	1,08	0,90	0,91	0,24	1,10	1,07	0,29	0,78	0,90	0,29
		400	0,10	0,76	0,90	0,68	0,75	0,24	0,90	0,91	0,29	0,78	0,72	0,29
		800	0,08	0,67	0,51	0,55	0,62	0,25	0,75	0,74	0,31	0,71	0,69	0,30
2	8	50	0,15	1,03	1,01	0,93	0,99	0,17	1,02	0,94	0,19	0,83	0,82	0,19
		100	0,16	0,94	1,00	0,81	0,88	0,17	0,95	0,95	0,12	0,87	0,75	0,18
		200	0,15	0,86	0,87	0,73	0,72	0,18	0,90	0,88	0,19	0,75	0,61	0,20
		400	0,14	0,64	0,81	0,43	0,58	0,16	0,78	0,68	0,20	0,72	0,37	0,20
		800	0,14	0,61	0,62	0,48	0,22	0,39	0,73	0,68	0,28	0,47	0,59	0,17
3	6	50	0,15	1,04	1,06	0,90	0,91	0,24	0,85	0,98	0,20	0,99	0,95	0,20
		100	0,16	0,85	0,99	0,84	0,85	0,14	0,79	0,98	0,20	0,91	0,91	0,21
		200	0,16	0,86	0,82	0,72	0,75	0,14	0,82	0,90	0,19	0,77	0,78	0,20
		400	0,16	0,77	0,77	0,59	0,62	0,15	0,80	0,90	0,19	0,70	0,73	0,18
		800	0,14	0,67	0,53	0,48	0,51	0,16	0,67	0,14	0,20	0,66	0,66	0,21
4	4	50	0,07	1,02	1,02	0,87	0,87	0,18	1,05	1,03	0,21	0,98	0,95	0,22
		100	0,16	0,90	0,93	0,79	0,84	0,16	0,93	0,96	0,22	0,89	0,86	0,21
		200	0,14	0,72	0,80	0,80	0,60	0,20	0,82	0,81	0,19	0,71	0,75	0,18
		400	0,16	0,86	0,62	0,41	0,62	0,27	0,73	0,73	0,21	0,70	0,53	0,21
		800	0,15	0,63	0,62	0,39	0,44	0,13	0,77	0,65	0,19	0,78	0,53	0,20
5	2	50	0,24	0,67	0,76	0,71	0,73	0,15	0,91	0,83	0,17	0,74	0,74	0,18
		100	0,14	0,74	0,75	0,64	0,67	0,32	0,55	0,78	0,15	0,72	0,69	0,17
		200	0,10	0,62	0,67	0,58	0,59	0,15	0,69	0,71	0,36	0,61	0,42	0,17
		400	0,13	0,59	0,48	0,49	0,48	0,15	0,63	0,68	0,16	0,62	0,50	0,15
		800	0,13	0,52	0,56	0,41	0,35	0,15	0,51	0,55	0,08	0,26	0,08	0,07

Ag, antigen; Ab, antibody (serum); B, blank (PBS); F1, serum from fish 1; F2, serum from fish 2.

TABLE C2. Antibody response (OD_{492}) to immunization for antigen optimization by ELISA using sonicated WB cells from strain AL20460 (atypical *A. salmonicida*) as antigen in ELISA at different concentrations.

Plate	Ag	Ab	Ag blocked with 3% skimmed milk						Ag not blocked					
No.	(µg/ml)	dilution	B	F1	F1	F2	F2	B	F1	F1	B	F2	F2	B
		50	0,23	1,18	1,17	1,06	1,22	0,24	1,31	1,31	0,26	1,23	1,21	0,27
		100	0,22	1,14	1,19	1,11	0,86	0,23	1,20	1,19	0,27	1,12	1,09	0,26
1	10	200	0,21	0,99	1,00	0,82	0,96	0,23	1,04	0,92	0,26	1,01	0,88	0,18
		400	0,22	0,85	0,82	0,71	0,66	0,23	0,75	0,89	0,26	0,85	0,83	0,27
		800	0,04	0,63	0,61	0,56	0,60	0,24	0,68	0,70	0,26	0,14	0,63	0,05
		50	0,11	1,17	1,16	1,05	1,07	0,16	1,06	1,15	0,21	1,00	1,06	0,19
		100	0,05	1,04	0,97	0,94	0,91	0,38	1,05	0,97	0,20	0,96	0,95	0,20
2	8	200	0,06	0,86	0,91	0,79	0,82	0,16	0,97	0,94	0,19	0,92	0,85	0,16
		400	0,05	0,70	0,71	0,64	0,61	0,15	0,79	0,77	0,20	0,75	0,70	0,16
		800	0,05	0,47	0,50	0,49	0,48	0,16	0,10	0,62	0,20	0,57	0,61	0,07
		50	0,14	1,06	1,10	0,97	0,95	0,14	1.158	1,16	0,89	0,91	0,96	0,18
		100	0,14	0,97	0,91	0,84	0,78	0,18	1,05	1,00	0,10	0,94	0,89	0,17
3	6	200	0,13	0,85	0,74	0,71	0,73	0,14	0,76	0,82	0,10	0,84	0,67	0,11
		400	0,14	0,65	0,64	0,58	0,59	0,14	0,72	0,70	0,09	0,70	0,68	0,19
		800	0,13	0,37	0,47	0,48	0,46	0,22	0,59	0,57	0,10	0,58	0,55	0,18
		50	0,10	0,92	0,95	0,78	0,83	0,10	0,99	0,96	0,15	0,89	0,73	0,16
		100	0,10	0,72	0,89	0,73	0,70	0,10	0,99	0,77	0,15	0,85	0,56	0,16
4	4	200	0,11	0,43	0,70	0,50	0,53	0,12	0,76	0,20	0,16	0,71	0,55	0,17
		400	0,10	0,54	0,50	0,49	0,27	0,11	0,34	0,61	0,16	0,61	0,56	0,16
		800	0,10	0,42	0,31	0,37	0,38	0,12	0,49	0,52	0,15	0,49	0,48	0,16
		50	0,09	0,42	0,32	0,54	0,53	0,07	0,57	0,57	0,13	0,38	0,62	0,08
		100	0,09	0,67	0,60	0,48	0,50	0,09	0,65	0,61	0,13	0,48	0,43	0,11
5	2	200	0,08	0,41	0,51	0,36	0,35	0,09	0,55	0,54	0,15	0,54	0,36	0,13
		400	0,09	0,29	0,42	0,31	0,30	0,09	0,45	0,39	0,15	0,46	0,29	0,13
		800	0,10	0,27	0,28	0,24	0,26	0,09	0,39	0,42	0,15	0,37	0,27	0,14

TABLE C3. Antibody response (OD_{492}) to immunization for antigen optimization by ELISA using sonicated WB cells from strain AL122 (*V. anguillarum* O2) as antigen at different concentrations.

Plate	Ag	Ab	Ag blocked with 3% skimmed milk						Ag not blocked					
no.	(µg/ml)	dilution	B	F1	F1	F2	F2	B	F1	F1	B	F2	F2	B
		50	0,17	1,80	1,69	1,66	1,85	0,19	1,95	1,77	0,23	1,85	1,83	0,24
		100	0,17	1,75	1,73	1,66	1,85	0,23	1,70	1,86	0,21	1,82	1,80	0,23
1	10	200	0,20	1,75	1,79	1,38	1,74	0,23	1,82	1,79	0,22	1,73	1,75	0,23
		400	0,16	1,66	1,63	1,63	1,50	0,21	1,71	1,68	0,22	1,40	1,65	0,21
		800	0,17	1,50	1,07	1,01	1,38	0,22	1,54	1,55	0,24	1,53	1,47	0,23
		50	0,15	1,77	1,77	1,38	1,84	0,11	1,58	1,86	0,20	1,88	1,81	0,20
		100	0,17	1,72	1,68	1,74	1,73	0,12	1,79	1,83	0,20	1,81	1,74	0,24
2	8	200	0,12	1,56	1,67	1,23	1,71	0,12	1,80	1,79	0,21	1,74	1,58	0,22
		400	0,15	1,58	1,73	1,50	1,48	0,14	1,78	1,66	0,20	1,42	1,59	0,21
		800	0,16	1,40	1,45	1,26	1,33	0,13	1,51	1,49	0,21	1,41	1,30	0,21
		50	0,16	1,63	1,74	1,71	1,72	0,16	1,74	1,74	0,22	1,51	1,71	0,22
		100	0,17	1,76	1,76	1,71	1,67	0,17	1,73	1,79	0,20	1,61	1,65	0,20
3	6	200	0,16	1,64	1,72	1,50	1,62	0,13	1,74	1,61	0,22	1,67	1,63	0,23
		400	0,16	1,43	1,39	1,42	1,43	0,13	1,66	1,61	0,21	1,33	1,46	0,23
		800	0,15	1,25	1,13	1,02	1,24	0,17	1,42	1,36	0,20	1,31	1,27	0,23
		50	0,16	1,42	1,69	1,70	1,67	0,13	1,76	1,68	0,19	1,69	1,56	0,19
		100	0,17	1,76	1,70	1,62	1,67	0,10	1,66	1,65	0,18	1,63	1,54	0,19
4	4	200	0,16	1,63	1,56	1,55	1,52	0,15	1,70	1,20	0,19	1,56	1,47	0,19
		400	0,15	1,54	1,47	1,38	1,33	0,15	1,53	1,51	0,18	1,34	1,32	0,19
		800	0,16	1,33	1,31	1,15	1,12	0,15	1,38	1,33	0,19	0,90	1,09	0,20
		50	0,14	1,38	1,48	1,53	1,62	0,12	1,67	1,61	0,21	1,66	1,57	0,17
		100	0,15	1,54	1,61	1,55	1,48	0,12	1,63	1,59	0,14	1,58	1,49	0,15
5	2	200	0,13	1,52	1,59	1,41	1,43	0,12	1,58	1,49	0,20	1,34	1,31	0,11
		400	0,14	1,34	1,14	1,15	1,20	0,12	1,34	1,36	0,21	1,00	1,20	0,20
		800	0,15	1,06	0,80	0,93	0,93	0,15	1,18	1,16	0,19	1,01	0,94	0,19

Table C4. Antibody response (OD_{492}) to immunization for antigen optimization by ELISA using sonicated WB cells from *Pasteurella* spp. as antigen at different concentrations.

Plate No.	Ag (µg/ml)	Ab Dilutions	Ag blocked with 3% skimmed milk						Ag not blocked					
			B	F1	F1	F2	F2	B	F1	F1	B	F2	F2	B
1	10	50	0,18	1,34	1,38	1,40	1,47	0,20	1,59	1,48	0,20	1,53	1,48	0,06
		100	0,16	1,29	1,33	1,25	1,23	0,20	1,44	1,41	0,25	1,51	1,49	0,09
		200	0,16	1,26	1,24	1,20	1,51	0,19	1,45	1,45	0,24	1,44	1,46	0,11
		300	0,16	1,12	1,17	1,23	1,19	0,19	1,35	1,40	0,24	1,22	1,23	0,12
		400	0,16	1,01	1,16	1,18	1,31	0,19	1,35	1,36	0,24	1,42	1,18	0,06
2	8	50	0,16	1,14	1,15	1,22	1,33	0,15	1,38	1,32	0,21	1,27	0,84	0,21
		100	0,14	1,11	1,11	1,20	1,18	0,13	1,26	1,29	0,21	1,22	1,34	0,21
		200	0,13	1,05	1,01	1,15	1,20	0,17	1,26	1,25	0,21	1,20	1,23	0,23
		300	0,16	0,98	1,00	1,07	1,22	0,17	1,20	1,19	0,21	1,03	1,35	0,21
		400	0,19	0,90	0,79	1,03	1,08	0,17	1,15	1,14	0,21	1,26	1,21	0,25
3	6	50	0,14	0,98	1,02	1,07	0,81	0,16	1,23	1,04	0,20	1,15	1,29	0,12
		100	0,14	0,89	0,82	1,05	1,19	0,09	1,17	1,18	0,19	1,28	1,19	0,18
		200	0,14	0,89	0,93	0,93	1,06	0,13	1,03	0,92	0,19	1,07	1,11	0,20
		300	0,14	0,94	0,88	0,96	1,00	0,13	1,00	1,00	0,19	1,18	1,08	0,21
		400	0,12	0,69	0,78	0,88	0,56	0,12	0,84	0,95	0,19	0,55	0,97	0,21
4	4	50	0,23	0,90	0,94	1,07	0,96	0,15	0,87	1,04	0,18	1,14	1,02	0,18
		100	0,13	0,84	0,93	0,91	1,09	0,18	0,78	1,02	0,18	1,11	1,00	0,19
		200	0,11	0,89	0,77	0,93	0,85	0,13	0,81	0,98	0,17	1,07	0,89	0,16
		300	0,12	0,78	0,60	0,84	0,79	0,13	0,86	0,91	0,19	1,00	0,92	0,19
		400	0,12	0,71	0,59	0,77	0,86	0,14	0,86	0,87	0,17	0,92	0,87	0,18
10	2	50	0,11	0,67	0,61	0,75	0,83	0,11	0,59	0,79	0,14	0,90	0,81	0,13
		100	0,11	0,68	0,58	0,73	0,77	0,11	0,60	0,75	0,13	0,86	0,87	0,12
		200	0,12	0,59	0,66	0,69	0,60	0,12	0,63	0,74	0,11	0,82	0,80	0,13
		300	0,12	0,61	0,52	0,58	0,61	0,12	0,71	0,67	0,14	0,74	0,75	0,13
		400	0,11	0,54	0,47	0,56	0,66	0,13	0,59	0,60	0,14	0,67	0,58	0,14

Table C5. Antibody response (OD_{492}) to immunization for antigen optimization by ELISA using sonicated WB cells from strain 8657 (*V. ordalii*) as antigen at different concentrations.

Plate	Ag	Ab	Ag blocked with 3% skimmed milk					Ag not blocked				
no	(µg/ml)	dilution	F1	F1	F2	F2	B	F1	F1	B	F2	F2
		50	1,09	1,10	1,12	1,02	0,17	1,15	1,15	0,19	1,14	1,13
		100	0,99	1,00	0,98	0,98	0,18	1,08	1,06	0,26	1,00	0,94
1	10	200	0,84	0,83	0,84	0,77	0,17	0,95	0,86	0,19	0,84	0,78
		400	0,62	0,77	0,66	0,67	0,19	0,72	0,67	0,18	0,29	0,62
		800	0,44	0,46	0,44	0,46	0,16	0,52	0,50	0,22	0,42	0,46
		50	1,11	1,15	1,13	1,16	0,18	1,19	1,19	0,21	1,19	1,17
		100	1,01	1,03	0,95	1,02	0,18	1,11	1,04	0,12	1,08	1,07
2	8	200	0,85	0,88	0,83	0,89	0,18	0,94	0,91	0,30	0,92	0,88
		400	0,68	0,64	0,69	0,69	0,18	0,77	0,65	0,21	0,71	0,69
		800	0,46	0,49	0,48	0,48	0,18	0,55	0,52	0,19	0,52	0,51
		50	1,23	1,15	1,23	1,19	0,16	1,07	1,10	0,20	1,06	1,23
		100	1,06	1,04	1,02	1,07	0,17	0,97	1,00	0,20	0,98	0,94
3	6	200	0,88	0,87	0,85	0,81	0,17	0,93	0,96	0,19	0,90	0,83
		400	0,67	0,66	0,59	0,64	0,17	0,79	0,74	0,20	0,68	0,64
		800	0,48	0,47	0,47	0,47	0,18	0,60	0,56	0,18	0,49	0,52
		50	1,07	1,09	1,12	1,13	0,16	1,10	1,10	0,19	0,86	0,95
		100	0,98	1,06	0,99	1,02	0,16	1,07	1,00	0,20	0,98	0,87
4	4	200	0,85	0,86	0,85	0,84	0,17	1,05	0,89	0,20	0,84	0,76
		400	0,66	0,66	0,63	0,63	0,17	0,74	0,78	0,19	0,61	0,59
		800	0,46	0,47	0,45	0,44	0,18	0,52	0,53	0,18	0,43	0,45
		50	0,87	0,85	0,94	0,94	0,15	1,02	0,96	0,17	0,93	0,87
		100	0,82	0,85	0,82	0,86	0,15	0,85	0,93	0,18	0,89	0,84
5	2	200	0,70	0,72	0,69	0,74	0,15	0,82	0,79	0,18	0,77	0,75
		400	0,57	0,58	0,52	0,57	0,15	0,68	0,65	0,18	0,62	0,61
		800	0,40	0,37	0,40	0,41	0,15	0,55	0,54	0,20	0,47	0,44

Table C6. Specific antibody levels two weeks post–immunization with formalin inactivated WB cells of strain 8546 (atypical *A. salmonicida*) detected by ELISA using homologous sonicated WB cells as antigens.

Plate No.	Sample	Twofold serum Dilution									
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
		OD (492 nm)									
1	F1	1,15	1,10	0,94	0,77	0,63	0,47	0,26	0,14	0,06	0,02
	F1	1,16	1,20	0,94	0,82	0,63	0,45	0,26	0,10	0,07	0,02
	F2	0,88	0,76	0,59	0,45	0,37	0,23	0,13	0,03	0,05	0,02
	F2	0,96	0,67	0,56	0,40	0,33	0,23	0,14	0,06	0,02	0,02
	C2	0,21	0,13	0,08	0,05	0,03	0,03	0,01	0,01	0,00	-0,01
	C2	0,19	0,10	0,07	0,05	0,04	0,03	0,01	0,01	0,00	-0,01
2	F3	1,01	0,91	0,59	0,53	0,34	0,23	0,14	0,09	0,00	0,01
	F3	1,03	0,94	0,59	0,57	0,41	0,27	0,17	0,15	0,02	0,00
	F4	0,96	0,66	0,58	0,41	0,25	0,18	0,13	0,09	0,07	0,00
	F4	0,86	0,76	0,52	0,40	0,25	0,18	0,13	0,06	0,03	0,00
	F5	1,04	0,86	0,71	0,55	0,40	0,49	0,16	0,08	0,04	0,00
	F5	1,07	0,92	0,58	0,48	0,34	0,18	0,14	0,06	0,03	0,00
3	F6	0,99	0,89	0,58	0,55	0,40	0,18	0,17	0,09	0,06	0,03
	F6	1,01	0,83	0,68	0,52	0,38	0,24	0,17	0,10	0,05	0,02
	F7	1,09	0,90	0,73	0,49	0,34	0,18	0,13	0,08	0,05	0,03
	F7	1,08	0,89	0,70	0,55	0,29	0,19	0,12	0,07	0,04	0,02
	F8	1,11	0,94	0,67	0,53	0,39	0,26	0,17	0,07	0,05	0,05
	F8	1,11	0,95	0,52	0,56	0,30	0,26	0,17	0,12	0,05	0,04
4	F9	1,20	1,18	0,96	0,81	0,65	0,47	0,30	0,06	0,10	0,07
	F9	1,25	1,23	1,00	0,78	0,52	0,51	0,31	0,19	0,11	0,03
	F10	1,21	1,02	0,97	0,87	0,64	0,41	0,25	0,15	0,10	0,06
	F10	1,18	1,14	0,99	0,90	0,61	0,40	0,25	0,14	0,09	0,05
	C1	0,50	0,39	0,27	0,20	0,11	0,06	0,02	-0,01	-0,03	-0,04
	C1	0,48	0,36	0,27	0,20	0,08	0,08	0,02	-0,01	-0,03	-0,03

Table C7. Specific antibody levels two weeks post–immunization with formalin inactivated WB cells of strain AL20460 (atypical *A. salmonicida*) detected by ELISA using homologous sonicated WB cells as antigens.

Plate No.	Sample	Twofold serum dilution									
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
		OD (492 nm)									
1	F1	1,19	1,01	0,83	0,69	0,43	0,29	0,18	0,10	0,07	0,06
	F1	1,16	1,11	0,83	0,63	0,44	0,30	0,18	0,12	0,06	0,02
	F2	1,01	0,75	0,68	0,51	0,35	0,27	0,15	0,06	0,03	0,02
	F2	1,01	0,86	0,67	0,51	0,37	0,10	0,15	0,05	0,04	0,01
	C2	0,15	0,14	0,12	0,11	0,08	0,07	0,04	0,05	0,03	0,01
	C2	0,17	0,16	0,15	0,13	0,09	0,02	0,06	0,04	0,00	0,04
2	F3	1,29	1,22	1,17	1,08	0,96	0,80	0,66	0,46	0,28	0,15
	F3	1,30	1,27	1,14	1,04	0,98	0,84	0,67	0,47	0,29	0,11
	F4	1,22	1,09	0,89	0,73	0,52	0,36	0,21	0,11	0,05	0,01
	F4	1,21	1,11	0,83	0,73	0,55	0,37	0,22	0,12	0,06	0,03
	C2	0,20	0,19	0,19	0,17	0,13	0,09	0,06	0,04	0,03	-0,01
	C2	0,21	0,20	0,18	0,17	0,13	0,10	0,09	0,05	0,05	-0,01
3	F5	1,05	1,00	0,87	0,81	0,67	0,66	0,49	0,31	0,19	0,10
	F5	1,10	1,03	0,89	0,83	0,85	0,70	0,51	0,31	0,18	0,10
	F6	1,07	0,95	0,84	0,69	0,51	0,36	0,20	0,10	0,06	0,02
	F6	1,20	1,02	0,85	0,70	0,52	0,33	0,21	0,11	0,06	0,02
	C2	0,21	0,20	0,20	0,18	0,13	0,09	0,07	0,06	0,05	0,03
	C2	0,22	0,19	0,23	0,19	0,15	0,12	0,09	0,06	0,05	0,04
4	F7	1,20	1,02	0,85	0,72	0,51	0,37	0,25	0,14	0,09	0,05
	F7	1,17	1,04	0,86	0,72	0,53	0,40	0,25	0,14	0,08	0,04
	F8	1,21	1,18	1,06	0,96	0,79	0,57	0,40	0,21	0,13	0,05
	F8	1,23	1,15	1,08	0,96	0,83	0,59	0,39	0,23	0,13	0,07
5	F9	1,21	1,10	0,99	0,92	0,75	0,57	0,35	0,20	0,12	0,06
	F9	1,22	1,11	1,04	0,90	0,74	0,58	0,37	0,18	0,13	0,07
	F10	1,31	1,18	1,08	0,97	0,79	0,60	0,38	0,25	0,05	0,08
	F10	1,35	1,23	1,11	1,00	0,83	0,60	0,39	0,20	0,07	0,10

Table C8. Specific antibody levels two weeks post–immunization with formalin inactivated WB cells of strain AL122 (*V. anguillarum* O2) detected by ELISA using heterogeneous sonicated WB cells of strain 8752 (*V. anguillarum* O1) as antigens.

Plate No.	Sample	Twofold serum dilution									
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
		OD (492 nm)									
1	F1	1,36	1,37	1,27	1,25	0,97	0,70	0,38	0,18	0,11	0,01
	F1	1,38	1,34	1,27	1,14	1,02	0,68	0,39	0,17	0,07	0,00
	F3	1,37	1,33	1,26	1,15	0,92	0,62	0,38	0,19	0,10	0,05
	F3	1,37	1,33	1,24	1,14	0,92	0,65	0,40	0,17	0,10	0,04
	C1	0,76	0,53	0,33	0,21	0,14	0,10	0,07	0,05	0,03	0,01
	C1	0,78	0,52	0,36	0,23	0,10	0,08	0,06	0,04	0,00	0,00
2	F4	1,39	1,30	1,12	0,84	0,44	0,23	0,11	0,07	0,03	0,03
	F4	1,40	1,30	1,11	0,92	0,47	0,22	0,12	0,03	0,03	0,03
	F5	1,37	1,28	1,18	0,97	0,63	0,36	0,16	0,10	0,07	0,05
	F5	1,41	1,33	1,16	0,92	0,34	0,35	0,16	0,08	0,08	0,03
	C2	0,11	0,06	0,04	0,01	0,00	-0,02	-0,03	-0,04	-0,10	-0,12
	C2	0,10	0,05	0,03	0,00	-0,01	-0,02	-0,03	-0,05	-0,08	-0,10
3	F6	1,04	0,70	0,44	0,34	0,19	0,07	0,05	0,01	0,02	0,02
	F6	1,03	0,74	0,45	0,35	0,18	0,10	0,03	-0,04	0,02	0,02
	F7	1,35	1,25	0,93	0,65	0,33	0,15	0,07	-0,07	0,03	0,02
	F7	1,36	1,22	0,94	0,62	0,29	0,15	0,05	0,06	0,03	0,01
4	F8	1,30	1,18	1,10	0,98	0,62	0,39	0,26	0,15	0,10	0,04
	F8	1,30	1,19	1,08	0,89	0,60	0,44	0,24	0,14	0,10	0,06
	F9	1,41	1,44	1,40	1,34	1,22	1,09	0,74	0,35	0,21	0,11
	F9	1,46	1,45	1,38	1,32	1,23	1,07	0,60	0,42	0,22	0,11
5	F10	1,41	1,40	1,30	1,38	1,34	1,19	1,01	0,62	0,34	0,14
	F10	1,44	1,40	1,38	1,37	1,34	1,21	0,98	0,58	0,38	0,14
	F11	1,20	1,05	0,76	0,66	0,35	0,22	0,18	0,11	0,07	0,05
	F11	1,20	1,07	0,78	0,62	0,36	0,22	0,17	0,11	0,07	0,05

Table C9. Specific antibody levels two weeks post–immunization with formalin inactivated WB cells of *Pasteurella* spp. detected by ELISA using homologous sonicated WB cells as antigens.

Plate No.	Sample	Twofold serum dilution									
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
		OD (492 nm)									
1	F1	1,40	1,36	1,25	1,19	1,09	0,98	0,85	0,65	0,45	0,20
	F1	1,41	1,35	1,26	1,19	1,10	0,97	0,86	0,65	0,45	0,18
	F2	1,52	1,54	1,44	1,38	1,24	1,21	1,07	0,97	0,89	0,74
	F2	1,54	1,53	1,45	1,37	1,25	1,21	1,05	0,96	0,98	0,73
	C1	0,74	0,48	0,33	0,19	0,16	0,09	0,08	0,01	0,02	0,02
	C1	0,75	0,38	0,32	0,19	0,12	0,09	0,05	0,03	0,02	0,00
2	F3	1,54	1,30	1,10	1,09	0,96	0,79	0,53	0,25	0,06	0,02
	F3	1,52	1,10	1,12	1,07	0,94	0,78	0,53	0,27	0,07	0,02
	F4	1,35	1,19	1,18	1,09	1,01	0,88	0,66	0,27	0,20	0,07
	F4	1,34	1,28	1,20	1,07	1,00	0,88	0,66	0,30	0,24	0,09
	C2	0,16	0,09	0,06	0,02	-0,02	-0,03	-0,08	-0,08	-0,07	-0,13
	C2	0,17	0,11	0,06	0,02	-0,04	-0,03	-0,08	-0,08	-0,07	-0,13
3	F5	1,45	1,45	1,31	1,25	1,13	1,02	0,89	0,71	0,55	0,51
	F5	1,43	1,45	1,31	1,26	1,13	0,95	0,88	0,72	0,54	0,50
	F6	1,42	1,38	1,23	1,19	1,01	0,91	0,67	0,44	0,37	0,18
	F6	1,38	1,29	1,22	1,19	1,03	0,95	0,66	0,46	0,33	0,19
4	F7	1,53	1,33	1,27	1,24	1,12	0,83	0,78	0,66	0,47	0,23
	F7	1,52	1,22	1,27	1,23	1,09	0,82	0,84	0,67	0,47	0,26
	F8	1,36	1,22	1,09	1,03	0,85	0,75	0,60	0,37	0,19	0,06
	F8	1,31	1,20	1,07	1,02	0,81	0,76	0,54	0,37	0,18	0,07
5	F9	1,14	1,06	0,99	0,91	0,79	0,67	0,46	0,19	0,10	0,01
	F9	1,15	1,04	1,00	0,91	0,80	0,66	0,43	0,21	0,10	0,01
	F11	1,26	1,18	1,08	0,96	0,87	0,74	0,55	0,33	0,11	0,02
	F11	1,22	1,19	1,09	0,97	0,87	0,73	0,60	0,32	0,12	0,01

Table C10. Specific antibody levels two weeks post–immunization with formalin inactivated WB cells of strain 8657 (*V. ordalii*) detected by ELISA using homologous sonicated WB cells as antigens.

Plate No.	Sample	Twofold serum dilution									
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600
		OD (492 nm)									
1	F1	1,06	0,84	0,57	0,35	0,20	0,11	0,07	0,04	0,03	0,02
	F1	1,00	0,83	0,57	0,34	0,20	0,13	0,06	0,03	0,02	0,02
	F2	0,53	0,32	0,17	0,09	0,06	0,06	0,03	0,02	0,02	0,01
	F2	0,53	0,30	0,17	0,10	0,06	0,04	0,04	0,02	0,02	0,01
	C1	0,74	0,58	0,41	0,24	0,13	0,07	0,03	0,03	0,02	0,02
	C1	0,73	0,59	0,40	0,27	0,14	0,07	0,04	0,03	0,04	0,03
2	F3	1,07	0,90	0,65	0,25	0,16	0,11	0,08	0,05	0,03	0,00
	F3	1,07	0,91	0,64	0,24	0,19	0,12	0,10	0,05	0,03	0,00
	F4	0,63	0,43	0,34	0,20	0,07	0,06	0,03	0,02	0,02	0,03
	F4	0,61	0,43	0,37	0,19	0,07	0,05	0,02	0,02	0,03	0,01
	C2	0,28	0,19	0,11	0,04	0,04	0,03	0,02	0,02	0,02	0,01
	C2	0,29	0,19	0,10	0,07	0,05	0,05	0,02	0,02	0,02	0,01
3	F5	1,18	0,99	0,79	0,55	0,34	0,21	0,13	0,08	0,04	0,03
	F5	1,10	1,03	0,77	0,57	0,33	0,22	0,12	0,05	0,05	0,01
	F6	0,93	0,64	0,39	0,23	0,12	0,07	0,04	0,02	0,01	0,02
	F6	0,92	0,63	0,38	0,23	0,34	0,07	0,04	0,01	0,01	0,02
4	F7	0,50	0,29	0,13	0,07	0,03	0,06	0,05	0,05	0,08	0,02
	F7	0,51	0,29	0,09	0,07	0,04	0,01	0,05	0,04	0,02	0,01
	F8	0,96	0,76	0,51	0,32	0,18	0,12	0,09	0,06	0,04	0,04
	F8	0,93	0,75	0,49	0,31	0,19	0,12	0,07	0,04	0,02	0,03
5	F9	1,09	0,79	0,55	0,44	0,36	0,34	0,32	0,31	0,31	0,31
	F9	1,06	0,77	0,54	0,43	0,36	0,34	0,33	0,31	0,31	0,29

Table C11. Antibody levels to formalin inactivated WB cells of strain 8546 (atypical *A. salmonicida*) when tested for cross-reactivity using sonicated WB cells of strain AL20460 (atypical *A. salmonicida*) as antigens in ELISA.

Antiserum 8546					Antiserum AL20460				
F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
1.11	1.22	1.39	1.44	1.23	1.40	1.15	1.13	1.03	1.20
1.31	1.20	1.41	1.43	1.24	1.38	1.12	1.12	1.03	1.21
F6	F7	F8	F9	F10	F6	F7	F8	F9	F10
1.27	1.29	1.32	1.26	1.43	1.09	1.03	1.21	1.25	1.26
1.27	1.28	1.32	1.27	1.43	1.10	1.07	1.23	1.26	1.26
C2	C2	Blank	Blank	Blank	C1	C1	Blank	Blank	Blank
0.48	0.49	0.25	0.26	0.27	0.60	0.61	0.25	0.26	0.27
0.47	0.48	0.25	0.26	0.26	0.45	0.59	0.28	0.27	0.25

Table C12. Antibody levels to formalin inactivated WB cells of strain AL20460 (atypical *A. salmonicida*) when tested for cross-reactivity using sonicated WB cells of strain 8546 (atypical *A. salmonicida*) as antigens in ELISA.

Antiserum 8546					Antiserum AL20460				
F1	F2	F3	F4	F5	F1	F2	F3	F4	F5
1.46	1.13	1.26	1.09	1.35	1.23	1.31	1.33	1.48	1.39
1.45	1.28	1.30	1.13	1.34	1.25	1.30	1.48	1.47	1.39
F6	F7	F8	F9	F10	F6	F7	F8	F9	F10
1.09	1.38	1.33	1.17	1.36	1.43	1.41	1.45	1.22	1.50
1.10	1.15	1.31	1.34	1.38	1.40	1.40	1.43	1.42	1.46
C2	C2	C2	C2	C2	C1	C1	Blank	Blank	Blank
0.50	0.54	0.52	0.54	0.55	0.67	0.65	0.31	0.31	0.31
0.51	0.52	0.53	0.55	0.55	0.70	0.67	0.31	0.33	0.31

Table C13. Antibody levels to formalin inactivated WB cells of strain AL20460 (atypical *A. salmonicida*) when tested for cross-reactivity using sonicated WB cells of strain 8546 (atypical *A. salmonicida*) as antigens in ELISA.

Antibody response to a homologous antigen						
F5	F6	F8	F10	F11	C2	Blank
1,647	1,37	1,569	1,549	1,632	0,425	0,119
1,643	1,355	1,544	1,572	1,67	0,413	0,141

Antibody response to a heterogeneous antigen						
F5	F6	F8	F10	F11	C1	Blank
1,469	1,271	1,5	1,478	1,623	0,937	0,263
1,515	1,257	1,537	1,418	1,642	0,901	0,298

Table C14. Antibody levels detected by ELISA in fish 35 days post challenge strain 8546 (atypical *A. salmonicida*).

Tank no.	Sample	Challenge	OD 492 (nm)			
			1/200 serum dilution		1/800 serum dilution	
1	k1-1	Non-cha-c	0,14	0,15	0,06	0,08
	k1-2		0,19	0,17	0,10	0,11
	k1-3		0,12	0,11	0,11	0,11
	k1-4		0,15	0,15	0,09	0,11
	k1-5		0,13	0,11	0,08	0,10
8	k8-1		0,09	0,15	0,09	0,10
	k8-2		0,11	0,09	0,07	0,08
	k8-3		0,05	0,06	0,06	0,06
	k8-4		0,07	0,05	0,08	0,08
	k8-5		0,08	0,08	0,08	0,08
5	k5-1ip	$^{++}$i.p.	0,31	0,29	0,12	0,12
	k5-2ip		0,05	0,06	0,18	0,16
	k5-3ip		0,06	0,06	0,06	0,07
	k5-1	$^{++}$Co. (1:1)	0,16	0,16	0,14	0,13
	k5-2		0,05	0,04	0,09	0,07
	k5-3		0,25	0,22	0,20	0,18
	k5-4		0,12	0,09	0,10	0,11
	k5-5		0,11	0,12	0,08	0,08
4	k4-1	$^{++}$Co. (1:1)	0,09	0,08	0,08	0,07
	k4-2		0,07	0,06	0,06	0,05
	k4-3		0,15	0,13	0,09	0,10
	k4-4		0,05	0,05	0,03	0,04
	k4-5		0,07	0,07	0,04	0,03
	k4-1ip	$^{++}$i.p.	0,08	0,08	0,07	0,05
	k4-2ip		0,11	0,11	0,08	0,07
2	k2-1	$^{++}$Bath	0,07	0,06	0,06	0,06
	k2-2		0,07	0,07	0,07	0,05
	k2-3		0,07	0,06	0,07	0,05
	k2-4		0,19	0,13	0,15	0,15
	k2-5		0,06	0,07	0,05	0,05
	k2-6		0,05	0,05	0,05	0,05

$^{++}$, 10^6 bacteria/ml; i.p., intraperitoneal; Non-cha-c, non-challenged control; 1:1, i.p. to Co. ratio.

Table C14 (Continues)

Tank	Fish	Challenge	OD (492 nm)			
			1/200 serum dilution		1/800 serum dilution	
7	k7-1	+Co. (1:1)	0,14	0,10	0,07	0,08
	k7-2		0,11	0,11	0,08	0,07
	k7-3		0,10	0,12	0,12	0,12
	k7-4		0,10	0,11	0,09	0,10
	k7-5		0,12	0,13	0,06	0,08
	k7-1ip	+i.p.	0,09	0,09	0,09	0,11
	k7-2ip		0,19	0,19	0,11	0,11
	k7-3ip		0,05	0,05	0,06	0,09
	k7-4ip		0,07	0,07	0,12	0,08
	k7-5ip		0,10	0,10	0,09	0,07
3	k3-1	+Co. (1:1)	0,10	0,02	0,10	0,08
	k3-2		0,11	0,10	0,07	0,08
	k3-3		0,10	0,09	0,08	0,07
	k3-4		0,08	0,08	0,08	0,08
	k3-5		0,15	0,15	0,12	0,11
	k3-1ip	+i.p.	0,09	0,09	0,11	0,09
	k3-2ip		0,11	0,09	0,09	0,07
	k3-3ip		0,12	0,12	0,11	0,08
	k3-4ip		0,10	0,09	0,12	0,10
	k3-5ip		0,05	0,05	0,07	0,08
6	k6-1	++Bath	0,12	0,12	0,07	0,09
	k6-2		0,03	0,06	0,07	0,07
	k6-3		0,06	0,07	0,07	0,07
	k6-4		0,11	0,10	0,07	0,06
	k6-5		0,10	0,07	0,07	0,06
	k6-6		0,13	0,12	0,09	0,09

+, 10^3 bacteria/ml;

Table 15C. Antibody levels detected by ELISA in fish 49 days post challenge with strain AL20460 (atypical *A. salmonicida*).

Sample	Challenge	OD (492 nm)			
		1/200 serum dilution		1/800 serum dilution	
D5 -1		0,16	0,16	0,14	0,14
D5 -2	$^{++}$i.p.	0,16	0,17	0,15	0,16
D5 -3		0,10	0,12	0,09	0,10
D5 -4		0,12	0,13	0,10	0,10
D5 -5		0,14	0,16	0,15	0,17
D5 -6		0,15	0,16	0,14	0,15
D5 -7		0,14	0,16	0,12	0,13
D5 -8	$^{++}$Co. (1:1)	0,14	0,14	0,14	0,14
D5 -9		0,19	0,20	0,17	0,16
D5 -10		0,14	0,15	0,15	0,15
D5 -11		0,13	0,14	0,15	0,13
K1 -1		0,10	0,09	0,05	0,09
K1 -2		0,09	0,09	0,08	0,09
K1 -3		0,11	0,10	0,06	0,07
K1 -4		0,12	0,11	0,08	0,08
K1 -5	Non-cha-c	0,11	0,14	0,06	0,08
K8-1		0,10	0,11	0,08	0,12
K8-2		0,14	0,14	0,07	0,08
K8-3		0,12	0,12	0,09	0,06
K8-4		0,12	0,13	0,07	0,07
K8-5		0,14	0,14	0,06	0,08

Table C16. Antibody levels detected by ELISA in fish 43 days post challenge with strain 8752 (*V. anguillarum* O1).

Sample	Challenge	OD (492 nm)			
		1/200 serum dilution		1/800 serum dilution	
i.p.1	$^{++}$i.p.	0,31	0,31	0,08	0,08
i.p.2		0,21	0,22	0,07	0,08
Co.1	$^{++}$Co. (1:1)	0,17	0,18	0,05	0,05
Co.2		0,13	0,13	0,06	0,03
Co.3		0,15	0,14	0,05	0,05
Co.4		0,12	0,11	0,05	0,06
Co.5		0,12	0,12	0,04	0,03
Co.6		0,17	0,18	0,07	0,06
Co.7		0,19	0,19	0,08	0,07
Co.8		0,15	0,11	0,07	0,10
Co.9		0,12	0,16	0,05	0,06
Co.10		0,17	0,18	0,05	0,06
Co.11		0,14	0,12	0,04	0,04
Co.12		0,16	0,17	0,07	0,04
Co.13		0,27	0,27	0,08	0,08
Co.14		0,13	0,13	0,04	0,04
Co.15		0,17	0,16	0,05	0,06
Co.16		0,12	0,13	0,03	0,04
Co.17		0,20	0,19	0,06	0,06
Co.18		0,15	0,13	0,05	0,05
Co.19		0,19	0,19	0,06	0,06
Co.20		0,21	0,20	0,06	0,07
B1	$^{++}$Bath	0,07	0,07	0,01	0,01
B2		0,12	0,11	0,03	0,06
B3		0,05	0,05	0,01	0,00
B4		0,10	0,11	0,04	0,04
B5		0,07	0,05	0,03	0,03
B6		0,08	0,09	0,02	0,03
B7		0,11	0,07	0,02	0,02
B8		0,09	0,08	0,02	0,01
B9		0,11	0,09	0,04	0,02
B10		0,10	0,11	0,03	0,02

Table C16 (Continues).

Sample	Challenge	OD (492 nm)			
		1/200 serum dilution		1/800 serum dilution	
C1	Non-cha-c (Placebo)	0,03	0,03	0,02	0,02
C2		0,03	0,04	0,01	0,01
C3		0,11	0,12	0,03	0,04
C4		0,06	0,07	0,02	0,02
C5		0,11	0,13	0,03	0,03
C6		0,08	0,07	0,00	0,01
C7		0,08	0,08	0,02	0,02
C8		0,08	0,08	0,03	0,02
C9		0,11	0,12	0,02	0,02
C10		0,11	0,11	0,00	0,01
C11		0,09	0,11	0,01	0,01
C12		0,14	0,17	0,04	0,03
C13		0,07	0,08	0,02	0,02
C14		0,06	0,07	0,04	0,05
C15	Non-cha-c	0,02	0,04	0,04	0,08
C16		0,05	0,05	0,08	0,12
C17		0,07	0,06	0,09	0,14
C18		0,04	0,04	0,04	0,07
C19		0,01	0,04	0,05	0,06
C20		0,04	0,04	0,03	0,06
C21		0,01	0,01	0,04	0,03
C22		0,04	0,02	0,03	0,09
C23		0,03	0,04	0,02	0,02
C24		0,05	0,06	0,06	0,06
C25		0,02	0,03	0,02	0,01
C26		0,11	0,04	0,04	0,01
C27		0,02	0,03	0,03	0,02
C28		0,04	0,06	0,05	0,06
C29		0,03	0,03	0,02	0,01

Table C17. Antibody levels detected by ELISA in fish 11 days post challenge with *Pasteurella* spp. by cohabitation in a 1:1 ratio to i.p. at four different concentrations.

Sample	Bacterial dilution in PBS for challenge	OD (492 nm)			
		1/200 dilution		1/800 dilution	
1	1/20	0,26	0,28	0,06	0,06
2		0,19	0,24	0,06	0,08
3		0,08	0,09	0,03	0,04
4		0,16	0,19	0,03	0,06
5		0,25	0,27	0,10	0,15
6		0,24	0,31	0,10	0,09
7		0,11	0,13	0,04	0,04
8		0,10	0,15	0,05	0,05
1	1/50	0,28	0,31	0,17	0,18
2		0,32	0,41	0,15	0,22
3		0,26	0,35	0,12	0,16
4		0,33	0,40	0,18	0,23
5		0,24	0,26	0,13	0,15
6		0,34	0,36	0,12	0,23
7		0,23	0,26	0,14	0,15
8		0,31	0,33	0,15	0,26
1	1/100	0,21	0,36	0,17	0,18
2		0,21	0,31	0,16	0,17
3		0,31	0,43	0,18	0,21
4		0,18	0,31	0,14	0,16
5		0,13	0,26	0,13	0,14
6		0,32	0,48	0,11	0,20
7		0,10	0,19	0,13	0,11
8		0,17	0,19	0,09	0,12
9		0,30	0,30	0,13	0,20
10		0,26	0,31	0,14	0,20
1	1/1000	0,33	0,34	0,15	0,18
2		0,29	0,32	0,14	0,21
3		0,43	0,51	0,12	0,24
4		0,20	0,22	0,11	0,11
5		0,27	0,42	0,14	0,13
6		0,28	0,31	0,14	0,17

Table C18. Antibody levels detected by ELISA in vaccinated fish 43 days post challenge with strain 8546 (atypical *A. salmonicida*).

Serum dilution	Vaccine	Sera Samples									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
		OD (492 nm)									
1/200.	AMM3	1,08	1,24	0,79	0,94	1,09	1,20	1,33	1,36	0,96	NA
		1,13	1,21	0,76	1,00	1,02	1,22	1,32	1,41	0,95	NA
	Trivalent	1,59	1,41	1,62	1,05	1,53	1,05	1,16	1,46	1,35	1,12
		1,60	1,42	1,58	1,07	1,50	1,10	1,19	1,47	1,33	1,14
	Monovalent	1,33	1,34	0,89	1,07	0,89	1,01	0,89	0,92	1,58	0,89
		1,35	1,31	0,86	1,07	0,88	1,00	0,88	0,91	1,55	0,91
	Non-vacc-cha (PBS)	0,71	0,56	0,74	0,52	0,64	0,60	0,58	0,57	0,61	0,61
		0,65	0,55	0,75	0,51	0,64	0,62	0,57	0,55	0,60	0,62
	Non-cha-c (Placebo)	0,29	0,29	0,28	0,31	0,30	0,30	0,31	0,31	NA	NA
		0,29	0,30	0,28	0,33	0,30	0,30	0,31	0,31	NA	NA
		0,30	0,31	0,31	0,34	0,33	0,33	0,30	0,33	NA	NA
		0,31	0,32	0,31	0,36	0,34	0,33	0,39	0,34	NA	NA
		0,28	0,30	0,29	0,34	0,31	0,31	0,31	0,30	NA	NA
		0,30	0,30	0,28	0,31	0,30	0,32	0,31	0,30	NA	NA
		0,29	0,29	0,29	0,32	0,34	0,31	0,31	0,30	NA	NA
		0,30	0,35	0,29	0,32	0,30	0,30	0,31	0,30	NA	NA
1/800.	AMM3	0,83	0,96	0,46	0,67	0,75	0,74	0,70	0,94	0,62	NA
		0,81	0,90	0,48	0,66	0,77	0,74	0,76	0,92	0,59	NA
	Trivalent	1,33	1,09	1,43	0,53	1,12	0,61	0,82	1,02	0,69	0,63
		1,31	1,06	1,40	0,50	0,95	0,64	0,79	1,26	0,72	0,63
	Monovalent	0,90	0,79	0,53	0,69	0,49	0,59	0,53	0,47	1,27	0,53
		0,88	0,81	0,51	0,62	0,54	0,53	0,56	0,51	1,22	0,52
	Non-vacc-cha (PBS)	0,48	0,43	0,49	0,43	0,48	0,43	0,44	0,38	0,40	0,39
		0,47	0,42	0,49	0,42	0,48	0,44	0,44	0,38	0,38	0,42
	Non-cha-c (Placebo)	0,29	0,29	0,31	0,31	0,30	0,33	0,31	0,32	NA	NA
		0,30	0,29	0,28	0,31	0,29	0,30	0,29	0,29	NA	NA
		0,28	0,29	0,28	0,29	0,29	0,29	0,28	0,28	NA	NA
		0,29	0,28	0,28	0,30	0,28	0,31	0,30	0,31	NA	NA
		0,30	0,29	0,29	0,31	0,28	0,30	0,29	0,28	NA	NA
		0,31	0,30	0,30	0,33	0,31	0,32	0,31	0,31	NA	NA
		0,30	0,28	0,30	0,36	0,36	0,31	0,31	0,30	NA	NA
		0,31	0,29	0,29	0,34	0,29	0,30	0,29	0,28	NA	NA
Blank		0,30	0,29	0,29	0,28	0,28	0,28	0,31	0,30	0,28	0,27
		0,28	0,30	0,30	0,28	0,28	0,28	0,31	0,30	0,29	0,28
		0,29	0,28	0,28	0,27	0,29	0,28	0,30	0,28	NA	NA
		0,28	0,28	0,31	0,29	0,29	0,28	0,28	0,28	NA	NA

NA, not available; Non-challenged control (placebo); Non-vacc-cha, Non-vaccinated challenged (injected with PBS); AMM3, Alpha Marine Micro 3. F1 – F10; individual sera samples.

Table C19. Antibody levels detected by ELISA in vaccinated fish 31 days post challenge with strain AL20460 (atypical *A. salmonicida*).

Serum dilution	Vaccine	Sera samples									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
		OD (492 nm)									
1/200	AMM3	0,84	1,52	0,53	0,98	0,91	0,90	1,31	0,68	0,75	0,95
		0,97	1,58	0,55	1,06	0,90	0,96	1,36	0,70	0,76	1,01
	Trivalent	0,77	0,68	0,79	0,84	0,74	0,87	0,71	0,86	0,86	NA
		0,76	0,72	0,83	0,84	0,75	0,89	0,73	0,83	0,87	NA
	Monovalent	0,93	0,75	0,81	1,13	0,80	0,80	1,43	0,96	1,16	1,41
		1,00	0,77	0,84	1,15	0,82	0,77	1,45	0,99	1,26	1,43
	Non-vacc-cha (PBS)	0,45	0,40	NA	NA	NA	NA	NA	NA	NA	NA
		0,45	0,42	NA	NA	NA	NA	NA	NA	NA	NA
	Non-cha-c (Placebo)	0,31	0,30	0,32	0,33	0,32	0,31	0,31	0,33	0,33	0,32
		0,30	0,30	0,31	0,32	0,35	0,32	0,31	0,33	0,32	0,32
1/800	AMM3	0,40	1,08	0,36	0,67	0,43	0,56	0,85	0,42	0,44	0,53
		0,42	1,08	0,37	0,68	0,43	0,60	0,87	0,41	0,48	0,58
	Trivalent	0,54	0,44	0,71	0,54	0,51	0,55	0,47	0,55	0,54	NA
		0,54	0,41	0,74	0,56	0,50	0,54	0,47	0,56	0,58	NA
	Monovalent	0,61	0,48	0,43	0,53	0,41	0,43	1,06	0,43	0,64	0,88
		0,61	0,52	0,42	0,54	0,42	0,43	1,04	0,43	0,67	0,87
	Non-vacc-cha (PBS)	0,34	0,31	NA	NA	NA	NA	NA	NA	NA	NA
		0,36	0,32	NA	NA	NA	NA	NA	NA	NA	NA
	Non-cha-c	0,28	0,30	0,29	0,31	0,30	0,31	0,32	0,30	0,30	0,30
		0,25	0,28	0,28	0,31	0,30	0,31	0,29	0,32	0,31	0,31
Blank		0,19	0,20	0,21	0,22	0,22	0,22	0,25	0,23	0,20	0,22
		0,20	0,22	0,20	0,20	0,19	0,20	0,19	0,21	0,21	0,21

Table C20. Antibody levels detected by ELISA in vaccinated fish 31 days post challenge with strain 8752 (*V. anguillarum* O1).

Serum dilution	Vaccine	Sera samples									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
		OD (492 nm)									
1/200	Trivalent	0,72	0,84	0,64	0,71	1,13	0,57	1,14	0,85	0,85	0,93
		0,72	0,83	0,64	0,70	1,14	0,57	1,12	0,84	0,86	0,96
	Monovalent	0,86	0,93	0,97	0,79	1,09	0,65	1,04	0,79	0,76	0,90
		0,90	0,93	0,98	0,76	1,11	0,65	1,06	0,79	0,76	0,89
	Non-vacc-cha (PBS)	0,39	0,37	0,42	0,44	0,50	0,46	0,60	0,47	0,41	0,44
		0,42	0,38	0,43	0,43	0,51	0,47	0,57	0,69	0,46	0,44
	Non-cha-c (Placebo)	0,30	0,27	0,30	0,28	0,28	0,29	0,31	0,31	0,32	0,31
		0,29	0,25	0,30	0,30	0,27	0,29	0,28	0,33	0,31	0,31
		0,29	0,26	0,29	0,28	0,27	0,28	0,29	0,29	0,30	0,32
		0,29	0,28	0,29	0,30	0,28	0,29	0,30	0,30	0,31	0,32
1/800	Trivalent	0,40	0,42	0,32	0,39	0,62	0,33	0,56	0,36	0,45	0,57
		0,41	0,43	0,32	0,38	0,61	0,35	0,56	0,38	0,45	0,58
	Monovalent	0,49	0,42	0,49	0,49	0,65	0,39	0,62	0,39	0,42	0,39
		0,49	0,42	0,49	0,48	0,68	0,37	0,60	0,41	0,41	0,40
	Non-vacc-cha (PBS)	0,34	0,30	0,32	0,33	0,35	0,34	0,37	0,32	0,34	0,33
		0,35	0,30	0,32	0,35	0,35	0,35	0,36	0,34	0,33	0,33
	Non-cha-c (Placebo)	0,32	0,28	0,30	0,28	0,29	0,30	0,30	0,28	0,29	0,27
		0,30	0,28	0,29	0,28	0,28	0,23	0,30	0,27	0,28	0,29
		0,29	0,26	0,27	0,27	0,27	0,28	0,31	0,27	0,28	0,28
		0,28	0,26	0,26	0,28	0,28	0,28	0,29	0,28	0,28	0,28
Blank		0,25	0,25	0,27	0,25	0,27	0,14	0,19	0,25	0,28	0,29
		0,27	0,25	0,27	0,28	0,27	0,26	0,26	0,25	0,27	0,26

Table C21. Ring diameters for purified lumpfish IgM (ng/µl) determined by SRID at six different concentrations.

IgM	Ring (precipitin) diameter (mm) with 11 replicates											
(ng/µl)	1	2	3	4	5	6	7	8	9	10	11	Mean (mm)
160	11.5	11.3	11.4	11.0	10.7	9.6	11.0	10.9	10.7	11.9	11.9	11.1
80	10.6	10.3	10.3	9.5	8.7	9.6	9.2	9.2	9.0	10.3	10.5	9.7
40	9.4	9.3	9.3	7.6	6.2	6.2	7.9	7.8	7.4	8.8	8.8	8.1
20	8.7	8.2	7.8	6.6	6.2	5.7	6.4	6.2	6.2	7.4	7.2	6.9
10	6.5	6.6	6.4	5.4	5.5	5.4	5.5	5.8	5.3	5.9	5.9	5.8
5	5.9	5.7	5.4	5.1	5.2	4.9	5.5	4.8	4.9	5.3	5.4	5.3

Table C22. Determination of total serum IgM (mg/ml) by SRID in the lumpfish two weeks post immunization.

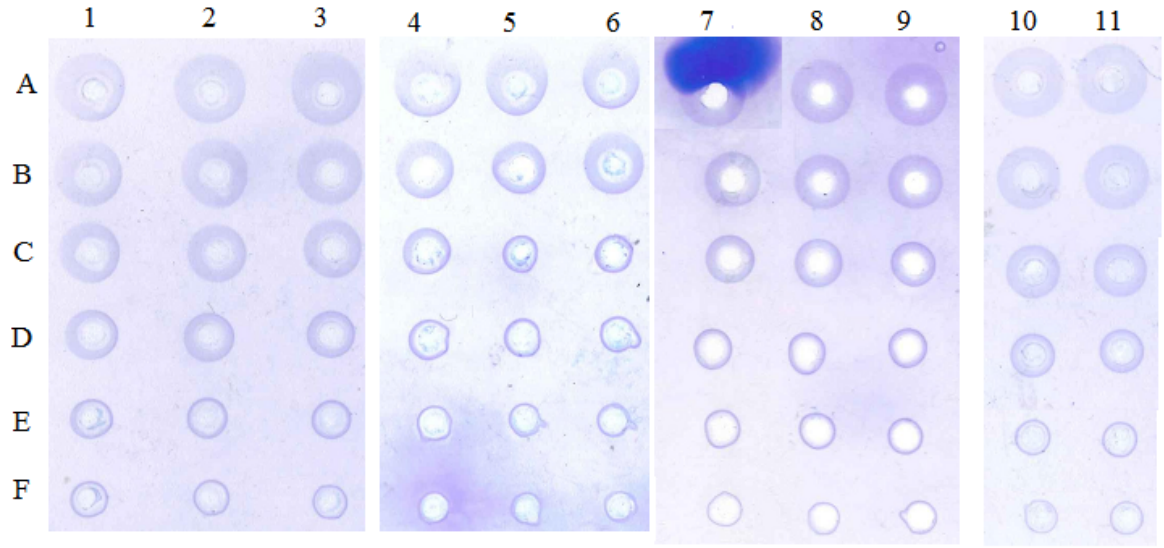
Group	Fish	Ring (precipitin) diameter (mm) with six replicates						Total serum (mg/ml)
		1	2	3	4	5	6	
8546	F1	9.36	9.36	NA	NA	NA	NA	1.28
	F2	8.63	8.62	NA	NA	NA	NA	0.84
	F3	8.47	7.58	NA	NA	NA	NA	0.59
	F4	7.88	7.58	NA	NA	NA	NA	0.50
	F5	7.88	7.28	NA	NA	NA	NA	0.46
	F6	9.32	9.38	NA	NA	NA	NA	1.27
	F7	9.96	9.96	NA	NA	NA	NA	1.82
	F8	8.34	7.94	NA	NA	NA	NA	0.63
	F9	7.15	7.18	NA	NA	NA	NA	0.36
	F10	6.88	6.71	NA	NA	NA	NA	0.29
AL20460	F1	8.98	9.22	9.27	NA	NA	NA	1.14
	F2	8.48	8.57	8.32	NA	NA	NA	0.76
	F3	8.03	7.73	7.87	NA	NA	NA	0.54
	F4	9.04	8.89	8.60	NA	NA	NA	0.95
	F5	7.63	7.38	7.03	NA	NA	NA	0.40
	F6	9.04	8.61	8.75	NA	NA	NA	0.93
	F7	9.19	8.61	8.46	NA	NA	NA	0.90
	F8	8.46	8.34	7.29	NA	NA	NA	0.59
	F9	8.01	8.13	8.03	NA	NA	NA	0.60
	F10	8.05	8.11	8.21	NA	NA	NA	0.62
AL122	F1	9.35	7.76	9.53	NA	NA	NA	0.97
	F2	9.53	9.53	9.17	NA	NA	NA	1.32
	F3	7.76	7.76	7.59	NA	NA	NA	0.49
	F4	7.94	6.88	6.88	NA	NA	NA	0.37
	F5	7.59	7.41	7.41	NA	NA	NA	0.43
	F6	7.59	7.41	7.23	NA	NA	NA	0.41
	F7	10.76	10.76	10.23	NA	NA	NA	2.61
	F8	10.76	10.05	9.35	NA	NA	NA	1.92
	F9	8.11	7.59	7.06	NA	NA	NA	0.46
	F10	10.05	9.17	8.47	NA	NA	NA	1.19

Table C23. Determination of total serum IgM (mg/ml) by SRID in small non-immunized lumpfish.

Fish	Rig diameter (mm)				Mean diameter (mm)	IgM (mg/ml)
F1	6,42	6,09	5,04	4,77	5,58	0,04
F2	7,41	6,09	7,42	7,80	7,18	0,09
F3	6,09	5,83	6,36	5,30	5,90	0,04
F4	9,20	9,30	9,70	9,68	9,47	0,34
F5	6,36	6,88	6,89	6,89	6,76	0,07
F6	6,25	5,83	7,15	6,89	6,53	0,06
F7	6,36	6,10	7,15	7,20	6,70	0,07
F8	7,68	7,15	8,80	8,21	7,96	0,14
F9	5,83	6,09	6,11	5,82	5,96	0,04
F10	9,07	9,00	9,00	9,00	9,02	0,26
F11	7,94	8,21	8,74	7,94	8,21	0,16
F12	5,30	5,80	6,04	6,04	5,80	0,04
F13	7,15	7,15	7,90	7,68	7,47	0,11
F14	8,48	8,21	9,30	9,27	8,82	0,23
F15	7,42	7,68	8,21	7,01	7,58	0,11
F16	9,70	9,50	9,73	9,79	9,68	0,39
F17	9,11	8,90	9,12	9,80	9,23	0,30
F18	8,97	8,48	8,74	8,47	8,67	0,21
F19	7,15	7,42	8,48	8,48	7,88	0,14
F20	6,36	6,18	6,40	6,62	6,39	0,06
F21	6,88	6,18	6,62	6,36	6,51	0,06
F22	7,75	7,41	8,74	8,48	8,10	0,15
F23	9,00	8,00	9,86	9,00	8,97	0,25
F24	6,09	6,35	6,80	6,62	6,47	0,06
F25	8,47	8,47	9,30	9,27	8,88	0,24
F26	7,42	7,68	9,20	9,00	8,33	0,18
F27	7,15	7,15	8,21	8,48	7,75	0,13
F28	6,62	6,62	6,62	6,89	6,69	0,07
				Mean	7,59 ± 1,23	0,14 ± 0,10

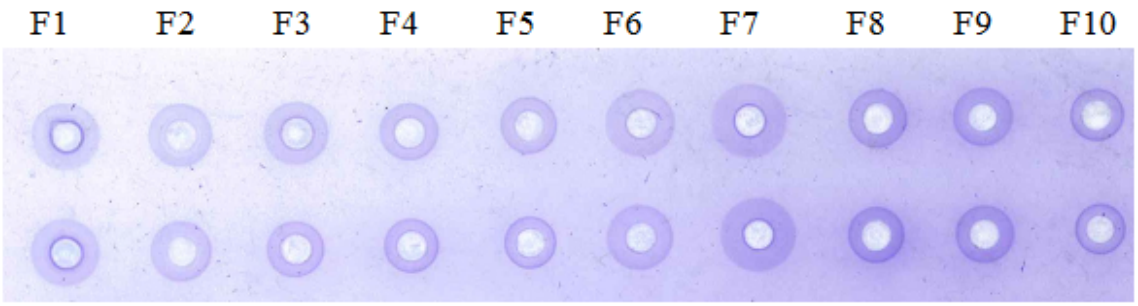
10. **APPENDIX D**. List of figures of results

Fig. D1. Purified lumpfish IgM at different concentrations used as a standard protein for making a standard curve to quantify total IgM in unknown serum samples by SRID.



A, 160ng/ml; B, 80ng/ml; C, 40ng/ml; D, 20ng; E, 10ng/ml; 5ng/ml; 1 -11, replicates.

Fig. D2. Quantification of lumpfish IgM by SRID two weeks post-immunization with formalin inactivated WB strains of 8546 (atypical *A. salmonicida*). Serum was diluted 1/20 in PBS.



F1 – F10, individual sera samples induplicates.

Fig. D3. Quantification of lumpfish IgM by SRID two weeks post-immunization with formalin inactivated WB strains of AL20460 (atypical *A. salmonicida*). Serum was diluted 1/20 in PBS.

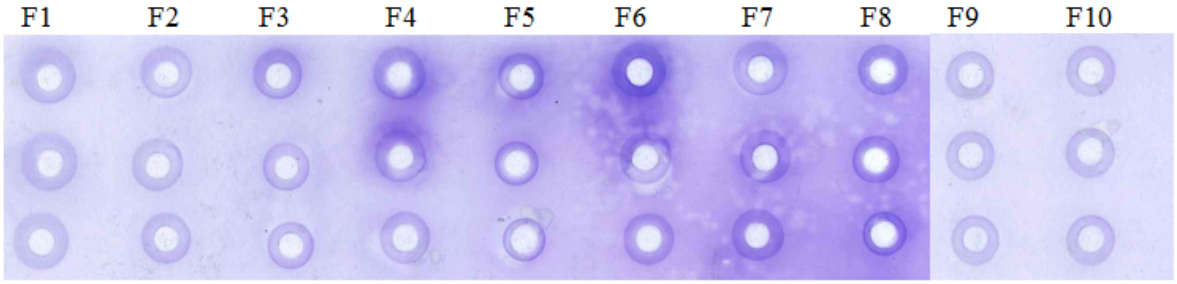


Fig. D4. Quantification of lumpfish IgM by SRID two weeks post-immunization with formalin inactivated WB strains of AL122 (*V. anguillarum* O2). Serum was diluted 1/20 in PBS.

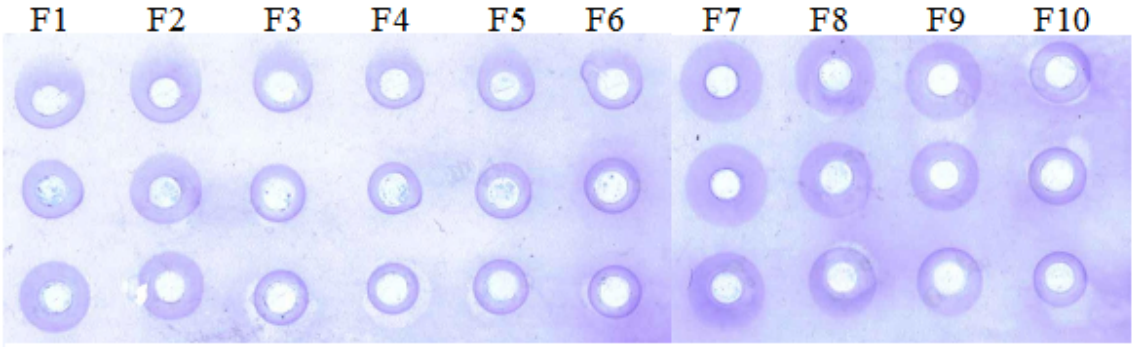


Fig. 5D. Quantification of lumpfish IgM by SRID two weeks post-immunization with formalin inactivated WB of *Pasteurella* - spp. Serum was diluted 1/20 in PBS.

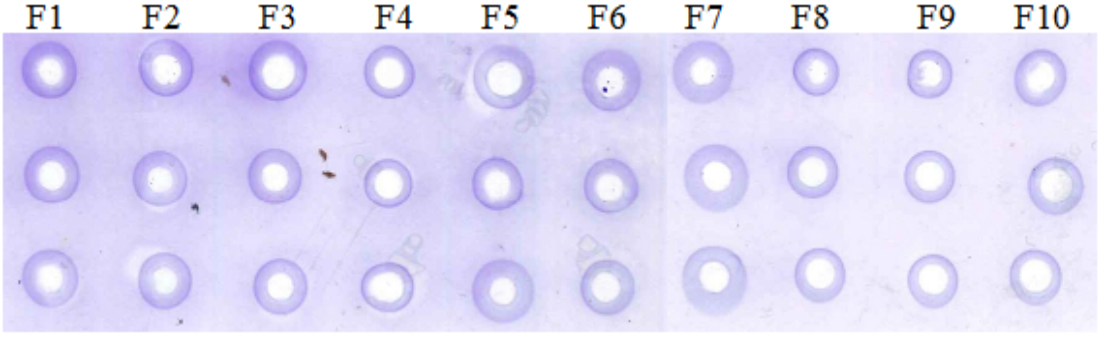


Fig. D6. Quantification of lumpfish IgM by SRID post-immunization against formalin inactivated WB of 8657. Serum dilution, 1/20 in PBS.

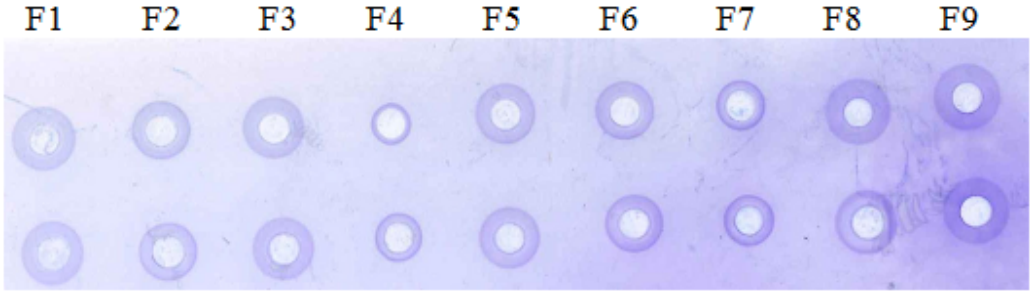
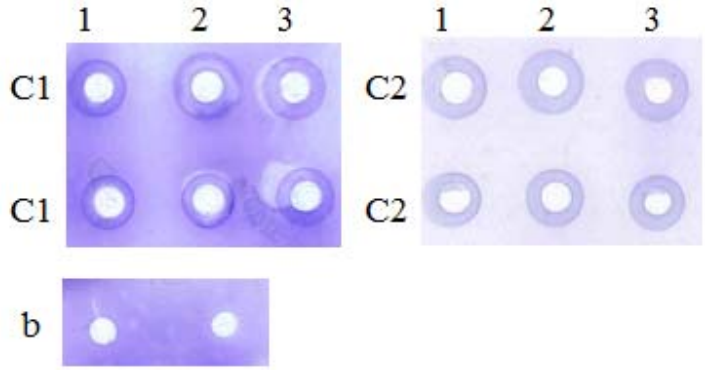
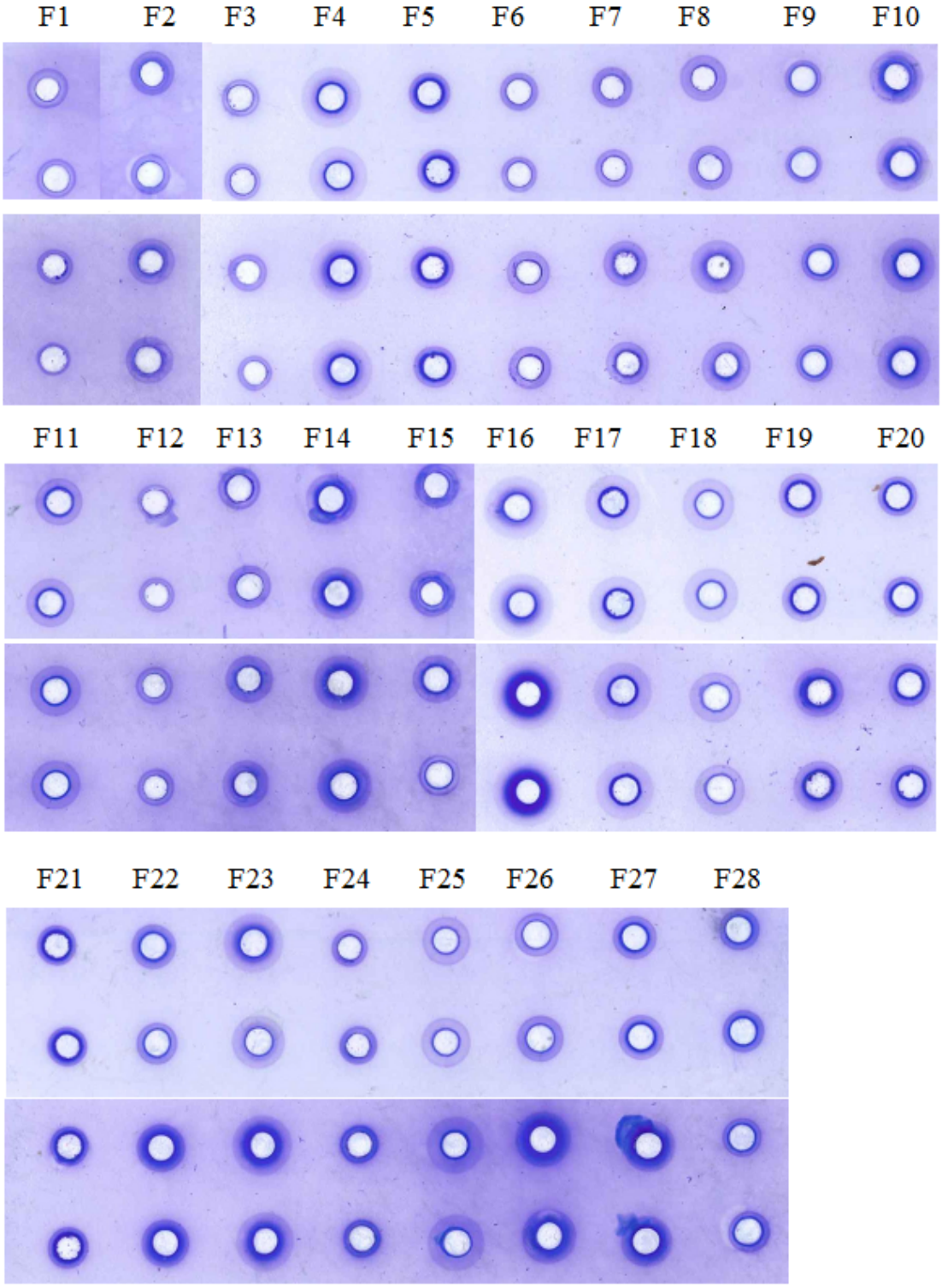


Fig. D7. Quantification of total serum IgM in non-immunized lumpfish by SRID. Serum dilution, 1/20 in PBS.



C1 – C2, sera samples in duplicates from non-immunized group; 1 – 3, replicates; b, blank.

Fig D8. Quantification of total serum IgM by SRID in small non – immunized lumpfish. Serum diluted 1/5 in PBS. F1 – F28 represent for individual sera samples in four replicates.



APPENDIX E. Publication

See next page for publication

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Functional characterization of IgM$^+$ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus* L.)

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ABSTRACT

The innate immune responses in lumpfish (*Cyclopterus lumpus* L.) have been shown to be functional, but little is currently known about the B cells, immunoglobulins or adaptive immune responses in this species. We have used anti-IgM antiserum to isolate B cells and compared them morphologically and functionally with other cell types. The fraction of IgM$^+$ cells among isolated peripheral blood leukocytes (PBL), head kidney leukocytes (HKL) and spleen leukocytes (SL) was in the range of 40%, 12% and 34%, respectively. The IgM$^+$ B cells had high phagocytic ability and were the predominant phagocytes in blood with higher capacity than IgM$^+$ B cells in HKL. Interestingly, among PBL, the most potent phagocytes were, in addition to monocytes, some small agranular uncharacterized IgM$^-$ cells. The IgM$^+$ B cells were positive for acid phosphatases (AcP), but negative for myeloperoxidase (MPO). Neutrophils were positive for MPO, while monocytes/macrophages and dendritic-like cells stained negatively. Monocytes/macrophages and the small, agranular IgM$^-$ cells stained most strongly positive for AcP corresponding to their high phagocytic capacity. Further, the ability to produce specific antibodies upon immunization verified adaptive immunity in the species.

The high proportion of phagocytic IgM$^+$ B cells and their phagocytic ability indicate a significant role of phagocytic B cells in lumpfish innate immunity. The present analyses also give strong indications that vaccination and immunostimulation of farmed lumpfish can be used to prevent disease and mortality caused by pathogenic organisms.

1. Introduction

In teleosts, three classes of immunoglobulins, IgM, IgD and IgT (called IgZ in zebrafish), have been identified. IgM is the predominant isotype and specialized in systemic immunity. IgT, which is present in low concentrations in serum, is an immunoglobulin specialized in gut mucosal immunity and is considered to be the functional equivalent to IgA in mammals and birds (reviewed in Zhang et al., 2011; Sunyer, 2013). The role of IgD in fish immunity is still unclear, but it has been suggested that it might function as an antigen binding receptor and play a role in immune responses as a mediator of innate immunity (Edholm et al., 2010).

In fish, two mutually exclusive B cell lineages exist based on the presence of either IgM or IgT (IgZ) (Zhang et al., 2010; reviewed in Zhang et al., 2011). The third class of immunoglobulin in fish, IgD, can be co-expressed with IgM on B cells, but not with IgT (Edholm et al., 2011; Wilson et al., 1997). Different subsets of B cells have been reported for different species: IgM$^+$/IgD$^+$/IgT$^-$, IgM$^-$/IgD$^+$/IgT$^-$ and IgM$^-$/IgD$^-$/IgT$^+$ in rainbow trout (Castro et al., 2014; Ramirez-Gomez et al., 2012; Zhang et al., 2010) and IgM$^+$/IgD$^-$, IgM$^+$/IgD$^+$ and IgM$^-$/IgD$^+$ in channel catfish (Edholm et al., 2010; reviewed in Salinas et al., 2011). Most fish species have IgT orthologs, such as zebrafish (Danilova et al., 2005), rainbow trout (Hansen et al., 2005), fugu (Savan et al., 2005), carp (Ryo et al., 2010; Savan et al., 2005; Xiao et al., 2010) and salmon (Tadiso et al., 2011). To date, catfish is the only species where an IgT equivalent has not been identified.

The unexpected discovery of professional phagocytic B cells in fish, amphibians and reptilians (Li et al., 2006; Øverland et al., 2010; Zhang et al., 2010; Zimmerman et al., 2010) has broadened the insight into B cell functions. Phagocytic B cells in fish (rainbow trout, salmon, cod and catfish) were identified by use of immunoglobulin specific antibodies to identify IgM$^+$ and IgT$^+$ cells. The fraction of IgM$^+$ phagocytes among isolated blood leukocytes in trout and salmon are higher compared with cod, but cod B cells have better phagocytic capacity shown by uptake of more particles per cell (Li et al., 2006; Øverland et al., 2010). In rainbow trout, the proportions of phagocytic IgT$^+$ cells, both from blood and head kidney, were

Abbreviations: MPO, myeloperoxidase; AcP, acid phosphatase; DC, dendritic-like cells; PBL, peripheral blood leukocytes; HKL, head kidney leukocytes; SL, spleen leukocytes; IgM, immunoglobulin M; SRID, single radial immunodiffusion.

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similar to that of IgM$^+$ cells, and up to seven beads were ingested by IgT$^+$ cells. The IgT$^+$ cells also had bactericidal abilities and were stimulated to proliferate and produce secreted Ig similarly to the IgM$^+$ cells upon bacteria encounter (Zhang et al., 2010). In fish, it is striking that the phagocytic B cells are the predominant phagocytic cells in blood (Li et al., 2006; Øverland et al., 2010). Although it has been reported that the professional phagocytic B cells in fish also have potent bactericidal abilities (Li et al., 2006; Zhang et al., 2010), their role in clearance of microorganisms and as antigen presenting cells to activate T cell responses remain to be elucidated.

The fundamental understanding of the role of B cells is their ability to be primed and activated to accomplish the adaptive specific immune response through immunoglobulin production. In teleosts, antibodies of the IgM class predominate in serum (Solem and Stenvik, 2006; Warr, 1997), but serum IgT and IgD have also been reported (reviewed in Sunyer, 2013). Secretory fish IgM is tetrameric, although a monomeric IgM has been reported in some species (Warr, 1995). The serum concentration of IgM varies between fish species (about 0.6–20 mg ml^{-1}), and depends on factors like developmental stage of the species, variations between individuals, environmental conditions and stress (Solem and Stenvik, 2006). For example, in cod (*Gadhus morhua* L.), IgM can constitute up to 50–70% of the total serum proteins in the range of 2–20 mg ml^{-1} and the amount of IgM in cod is high compared with salmon (Israelsson et al., 1991; Magnadottir, 1998; Magnadottir et al., 1999, 2009; Melingen and Wergeland, 2000). The understanding of the role of the natural IgM antibodies in fish is in its infancy, but in goldfish and trout natural antibodies have shown to be involved in defense against both viral and bacterial infections (Castro et al., 2013). Natural antibodies in cod have also broad specificity (Gonzalez et al., 1989; Magnadottir et al., 2009; Sinyakov et al., 2002). In rainbow trout, IgT in serum is present as a monomer while the molecular weight indicated a tetramer when isolated from mucus (Zhang et al., 2010). At protein level, IgD has only been described in two teleost species, catfish and trout, where it is monomeric (Edholm et al., 2011; Ramirez-Gomez et al., 2012). The ability of teleosts to produce specific antibodies upon vaccination varies. This is exemplified by Atlantic salmon demonstrating a high specific antibody response upon vaccination while cod has a low response, possibly due to the lack of MHC II (Pilstrøm et al., 2005; Star et al., 2011).

Professional phagocytic B cells have so far been shown in four fish species, belonging to *Salmoniformes*, *Gadiformes* and *Siluriformes*. In the present study, B cells in an evolutionary distant group, *Scorpaeniformes*, represented by lumpfish (*Cyclopterus lumpus* L.), have been characterized. We have analyzed the proportion of lumpfish IgM$^+$ B cells and their phagocytic ability and capacity using flow cytometry. Phenotypic and morphological characterization of sorted IgM$^+$ B cells has been performed by cytochemical staining and transmission electron microscopy (TEM) and the B cells were compared with other leukocyte subsets. Further, serum IgM protein has been characterized, and we have measured the concentration of total IgM and specific antibodies in serum from immunized fish.

2. Materials and methods

2.1. Fish and rearing conditions

Farmed lumpfish were provided by Fjord Forsk Sogn AS, Norway and kept in a 500 l tank in the rearing facilities at the Industrial Laboratory (ILAB) at Bergen High-Technology Centre under normal optimal rearing conditions, with a temperature of 12 °C, salinity of 34 ‰ and a photoperiod light/dark of 12:12 hrs. The oxygen saturation in the outlet water was a minimum of 77% air saturation. The fish were fed with commercial dry feed Amber Neptune, marine feed for gadoids, obtained from Skretting, Norway. There were no signs of infection and no mortality among the fish. The average weight and length of the fish were 13.2 ± 5 g and 5.7 ± 0.9 cm, respectively. Immunized fish had an average weight of 300 g (see Section 2.5).

2.2. Purification of lumpfish IgM

Purification of IgM from lumpfish serum was performed by gel-filtration and anion exchange chromatography using the Pharmacia FPLC chromatographic system (Pharmacia). The serum was obtained from wild caught fish with an approximate weight of 1 kg.

2.3. Production of rabbit anti-lumpfish IgM serum

Rabbit immune serum (anti-IgM) was produced by a Speedy 28-day protocol with four immunizations (Eurogentec, Belgium). Serum sampled from the rabbits before immunization is hereafter termed pre-serum. Specificity of the rabbit antisera were verified by ELISA and immunoblotting (Supplementary Fig. S1), in addition to immunostaining of isolated leukocytes from peripheral blood.

2.4. ELISA for evaluation of the reactivity of rabbit anti-lumpfish IgM sera

The reactivity of rabbit anti-lumpfish IgM sera was tested by ELISA using purified lumpfish IgM as antigen. Immunoplates (F96 Maxisorp, Nunc) were coated with 1 μg ml^{-1} purified IgM diluted in PBS by adding 150 μl per well. Wells where PBS replaced serum were used as blank. Skimmed milk (3% in PBS-T (PBS containing 0.05% Tween)) was used as blocking solution. The conjugate, horseradish peroxidase conjugated polyclonal goat anti-rabbit Ig (Dako, Denmark), was diluted 1:2000 in PBS-T. The OD was read at 492 nm in a Sunrise microplate reader (Tecan Group Ltd.) using Magellan software. Two parallels were performed for all dilutions.

2.5. Production of polyclonal antisera in lumpfish against lumpfish bacterial pathogens

For production of anti-bacteria immune sera, farmed lumpfish were kept at ILAB under the same rearing conditions and feed as described earlier. There were no signs of infection and no mortality among the fish. Antisera against the bacteria *Vibrio ordalii*, *V. anguillarum*, two strains of atypical *Aeromonas salmonicida*, and a *Pasteurella*-like bacteria (all bacteria isolated from outbreaks of disease in lumpfish), were prepared in ten lumpfish for each bacterial strain. For immunization 100 μl formalin-inactivated bacterial suspensions were harvested in the late exponential growth phase and were injected intraperitoneally. All fish were immunized three times. Non-immunized controls were fish injected with PBS. For the first injection the antigens were emulsified in Freud's incomplete adjuvant. For the following injections the antigens were diluted 1:2 in PBS 4 and 8 weeks after the first injection. The lumpfish were bled 2 weeks after the final injection. Sera fractions were collected and stored at –20 °C.

2.6. ELISA for detection of lumpfish antibodies to bacteria

Sera from lumpfish immunized with bacteria were analyzed by ELISA using bacteria as antigen. Immunoplates (F96 Maxisorp, Nunc) were coated with 10 μg ml^{-1} of bacteria diluted in PBS by adding 150 μl per well. Wells where PBS replaced serum were used as blank. Sera, diluted 1:50–1:25,600, were used as primary antibody. As no commercial anti-lumpfish conjugates exist, a secondary antibody was included. The secondary antibody used was rabbit anti-lumpfish IgM (diluted 1:1000) described in Section 2.3. The conjugate was peroxidase conjugated polyclonal goat anti-rabbit Ig (Dako Denmark) diluted 1:1000 in PBS-T. The OD was read at 492 nm in

a Sunrise microplate reader (Tecan Group Ltd.) using Magellan software. Two parallels were performed for all dilutions.

2.7. Determination of IgM in plasma by single radial immunodiffusion (SRID) assay

The concentration of total IgM from non-immunized and immunized fish was determined by SRID assay, also called the Mancini method, as described previously (Melingen and Wergeland, 2000) using three parallels for each dilution. Purified IgM was used to make the standard curve.

2.8. Size determination of serum IgM by gel filtration

One milliliter serum was applied to a 26/60 GE Healthcare gel filtration column in-house packed with superpose 6 prep grade media (Pharmacia LKB, Biotechnology AB) pre-equilibrated with buffer containing 50 mM Bis–Tris, pH 6.4 and 0.1 M NaCl. The column was run at 20 °C. Gel filtration HMW calibration kit (GE Healthcare) was used to make the standard curve. Fractions of 1 ml were collected and analyzed by SDS–PAGE and immunoblotting.

2.9. SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Purified IgM and PBL were fractioned on a 10 or 12% SDS–polyacrylamide gel using a Mini Protean Tetra Cell (Bio-Rad) and were heat-treated (96 °C for 5 min) in sample-buffer containing β-mercaptoethanol. For the oligomer analysis, serum fractions and purified IgM were heat-treated as earlier, but under non-reducing conditions (absence of β-mercaptoethanol). Samples of 10 μl (5×10^5 cells) were loaded onto each well, electrophoresed at 190 V for 45 min, followed by staining of proteins using Silver Stain Plus kit (Bio-Rad) or electroblotting onto 0.45 μm nitrocellulose membranes (Bio-Rad). Purified IgM from lumpfish was included as a positive control. Immunoblot analyses were performed using rabbit anti-lumpfish IgM serum (1:1000), polyclonal goat anti-rabbit immunoglobulins/HRP (Dako) (1:2000), developed with HRP Conjugate substrate kit (Bio-Rad) or Clarity Western ECL substrate (Bio-Rad) and visualized in Bio-Rad molecular image chemi Dic XRS+ Imaging system. Pre-serum as primary antibody was included as a negative control. The molecular weight of the heavy and light chains of IgM was calculated in Image lab software version 5.1 BETA (Bio-Rad) using precision plus protein Kaleidoscope prestained standard (Bio-Rad) as a molecular marker.

2.10. Tissue sampling and isolation of leukocytes

The fish were quickly netted and killed by a sharp blow to the head. Leukocytes were isolated from peripheral blood, head kidney and spleen using discontinuous Percoll gradients as described previously (Haugland et al., 2012a). Isolated leukocytes were counted in a CASY-TT Cell Counter™ (Innovatis AG) and viability and aggregation factors were determined for all samples.

2.11. Cytochemistry

Cytospin preparations were prepared by centrifugation of 100 μl of cell suspension ($1–1.5 \times 10^6$ cells ml^{-1}) at 1000 rpm for 3 min in a Shandon Cytospin 3 cytocentrifuge (Shandon Scientific Ltd.). The samples were air dried for 20 hrs at room temperature prior to cytochemical and immunofluorescence staining. The preparations of cells were stained using Colorrapid (Lucerna-Chem AG) according to the manufacturer's instructions.

2.11.1. Myeloperoxidase (MPO)

Staining was performed as described previously (Haugland et al., 2012a) by use of SIGMA*FAST* DAB tablets (Sigma-Aldrich) according to the manufacturer's instructions.

2.11.2. Acid phosphatase (AcP)

Staining of leukocytes on cytospun slides was performed using acid phosphatase kit (Sigma-Aldrich) according to the manufacturer's instructions. Two staining solutions were prepared, one with and one without added tartrate.

2.12. Immunofluorescence staining of cytospun leukocytes

Cytospin slides were fixed in acetone:methanol (1:1) for 90 s, washed once in PBS, pH 7.3 bath and incubated in a humidity chamber in the dark with PBS, pH 7.3, containing 0.5% (w/v) BSA for 30 min to block background staining. The slides were incubated for 1 h in a humidity chamber in the dark with primary antibody, rabbit anti-lumpfish IgM, diluted 1:1000 in PBS containing 0.5% (w/v) BSA, and washed twice in PBS, pH 7.3 bath, prior to incubation with Alexa Fluor® 488 $F(ab')_2$ fragments of goat anti-rabbit IgG (5 μg ml^{-1}) (Invitrogen) for 45 min and 3 washes in PBS bath. The slides were cover slipped with ProLong® Gold antifade reagent with DAPI (Invitrogen). For negative control, the rabbit pre-serum was used as primary antibody (1:1000).

2.13. Flow cytometry of leukocytes labeled with rabbit anti-lumpfish IgM serum

Peripheral blood leukocytes (PBL), spleen leukocytes (SL) and head kidney leukocytes (HKL) were labeled with rabbit anti-lumpfish IgM serum (1:10,000). Cells incubated without antibodies were used as control for auto-fluorescence and cells incubated with conjugate only were included to detect any non-specific binding. Pre-serum as primary antibody was included as a negative control for all leukocyte samples and used to define positive cells (2%). Alexa Fluor® 647 $F(ab')_2$ fragments of goat anti-rabbit IgG (5 μg ml^{-1}) (Invitrogen, Molecular Probes) (1:400) was used as secondary antibody and 10,000 cells were recorded in each sample. Cells were analyzed for forward scatter (FSC) and sideward scatter (SSC) patterns, representing the size and granularity of the cells, respectively, and for red fluorescence (FL4, long pass filter 670). Gating of the cells, defined by propidium iodide (PI, Sigma), was performed to exclude dead cells and debris from subsequent analyses. Staining with PI was performed according to the manufacturer's instructions and measured in FL2 (yellow–orange fluorescence, band pass filter 585/42). The analyses were performed on a BD FACSCalibur flow cytometer (BD Biosciences) equipped with a 488 nm argon laser and a 635 nm red diode laser using Cell Quest version 3.1 software (Becton Dickinson). Further data analyses were performed using FCS express 3 (De Novo Software).

2.14. Phagocytosis and labeling of IgM^+ cells

In the phagocytic assay, isolated leukocytes from peripheral blood and head kidney (5×10^6) cells in a total volume of 0.5 ml L-15 + medium in 24 well culture plates (Nunc) were incubated with fluoresbrite carboxylate YG 1.0 micron microspheres (Polysciences) as described previously (Haugland et al., 2012a), but with a cell/bead ratio of 1:20 and incubation for 5 hrs at 15 °C. Negative controls were wells containing leukocytes, but no beads. Following incubation, non-adherent cell suspension were collected and mixed with adherent cells that were carefully loosened using trypsin–EDTA (Lonza BioWhittaker). Non-ingested beads were removed by placing the cell-bead suspension at the top of a glucose-cushion consisting of

3 ml PBS, pH 7.3 with 3% (w/v) BSA and 4.5% (w/v) D-glucose and centrifugation at 84 × *g* for 10 min at 4 °C. The cell pellets were re-suspended in 500 µl L-15 + medium. The leukocyte suspensions were left over night at 4 °C prior to immunolabeling allowing the cells to recover after phagocytosis.

Further, the resuspended cells were labeled using polyclonal anti-IgM serum (1:10,000) as primary antibody and Alexa Fluor® 647 $F(ab')_2$ fragments of goat anti-rabbit IgG (5 µg ml^{-1}) (Invitrogen, Molecular Probes) (1:400) as secondary antibody prior to analysis with a BD FACSCalibur flow cytometer or sorting with a BD FACS Aria II flow cytometer. Negative controls for phagocytosis were cell suspensions incubated without beads while negative controls for IgM were cell suspensions incubated with rabbit pre-serum instead of primary antibody. Cells were analyzed for forward scatter (FSC) and sideward scatter (SSC) patterns, representing the size and granularity of the cells, respectively, for yellow–green bead fluorescence (FL1) and red fluorescent (FL4) detecting IgM^+ cells. The samples were analyzed as described in Section 2.13.

2.15. Cell sorting of PBL

PBL was labeled with rabbit polyclonal anti-IgM serum as described earlier and sorted in a BD FACS Aria II flow cytometer (Becton Dickinson).Three fractions were collected; IgM^+ cells, small IgM^- cells and large IgM^- cells. Following phagocytosis and IgM labeling, IgM^+ and IgM^- phagocytes from blood were sorted, cytospun and stained with Colorrapid.

2.16. Transmission electron microscopy (TEM)

FACS sorted cell fractions (IgM^+, large and small IgM^- cells from blood and IgM^+ and IgM^- phagocytes from blood and head kidney) were prepared for TEM at MIC, UiB. The samples were fixed in 1.5–2% glutaraldehyde in 0.1 M sodium cacodylate buffer and post-fixated in 1% osmium tetraoxide prior to embedding in Agar 100 resin. Thin sections (50–60 nm) were stained with uranyl and lead. The preparations were analyzed at MIC, UiB, using the transmission electron microscope Joel JEM-1230.

2.17. Digital imaging and image processing

All cytospin slides were examined in a Zeiss Axioskop 2 plus microscope (Carl Zeiss) and photographs of the cells were captured using a Nikon DS camera Head DS-5M. The pictures were processed in Adobe Photoshop CS5 (Adobe Systems Incorporated).

2.18. Statistics

Two-tailed unpaired t tests were performed for all analyses using GraphPad Prism 5. The results were considered significant when $P < 0.05$. In Prism 5, $P > 0.05$ = n.s., $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$.

3. Results

3.1. IgM in serum is a tetramer

In order to characterize lumpfish IgM at the protein level, IgM, purified from serum, was applied to SDS–PAGE. The Mw of the heavy and light chain was determined to be 80.8 kDa and 25.8 kDa, respectively (Fig. 1A). To determine if IgM is an oligomer, purified serum IgM was boiled under non-reducing conditions prior to electrophoresis to avoid breaking of inter-monomeric bonds. IgM had high Mw, showing that it is not a monomer (Fig. 1A). The oligomeric form of IgM in serum was determined with gel filtration using HMW calibration kit (Fig. 1B). IgM, detected by rabbit anti-lumpfish IgM polyclonal serum, eluted with a peak at 49 ml, corresponding to a Mw of approximately 760 kDa, suggests that serum IgM is a tetramer (Fig. 1B).

3.2. IgM^+ B cells are most abundant among peripheral blood and spleen leukocytes

The rabbit anti-lumpfish IgM polyclonal serum also reacted with leukocytes in immunoblotting (Supplementary Fig. S1B, C) and with live cells in flow cytometer. The percentage of IgM^+ B cells among isolated leukocytes from peripheral blood, head kidney and spleen could thus be determined using flow cytometry. The IgM^+ B cells,

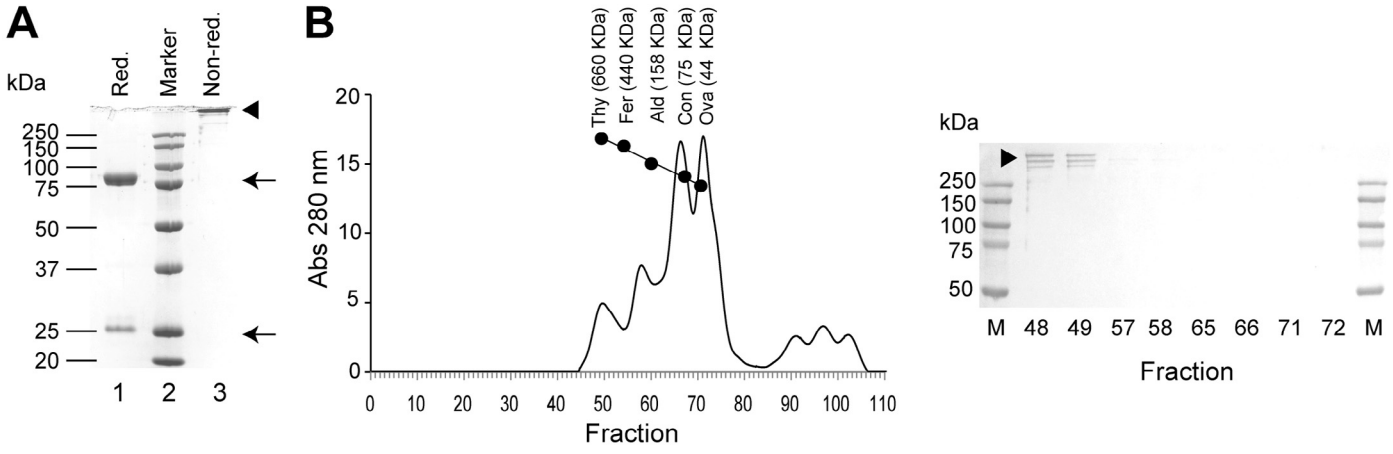

Fig. 1. Lumpfish serum IgM is a tetramer. (**A**) 12% SDS–PAGE of IgM purified from serum. Lane 1, IgM boiled under reducing conditions prior to SDS–PAGE; lane 2, kaleidoscope prestained marker; lane 3, IgM boiled under non-reducing conditions (absence of β-mercaptoethanol) prior to SDS–PAGE. Arrows = heavy and light chain of immunoglobulin, arrowhead = polymeric immunoglobulin. (**B**) Elution profile of lumpfish serum in gel filtration column. *Left panel*: The elution volumes for the proteins in the calibration kit are shown and the sizes of the proteins are given in the parentheses. Ovalbumin (Ova), Conalbumin (Con), Aldolase (Ald), Ferritin (Fer) and Thyroglobulin (Thy). *Right panel*: Two fractions from each peak of the serum-samples were resolved with 10% SDS–PAGE and immunoblotted using polyclonal anti-lumpfish IgM serum. The numbers below the immunoblot show the selected fractions. M = molecular marker. Arrowhead = polymeric IgM. The elution profile is representative for four different sera analyzed.

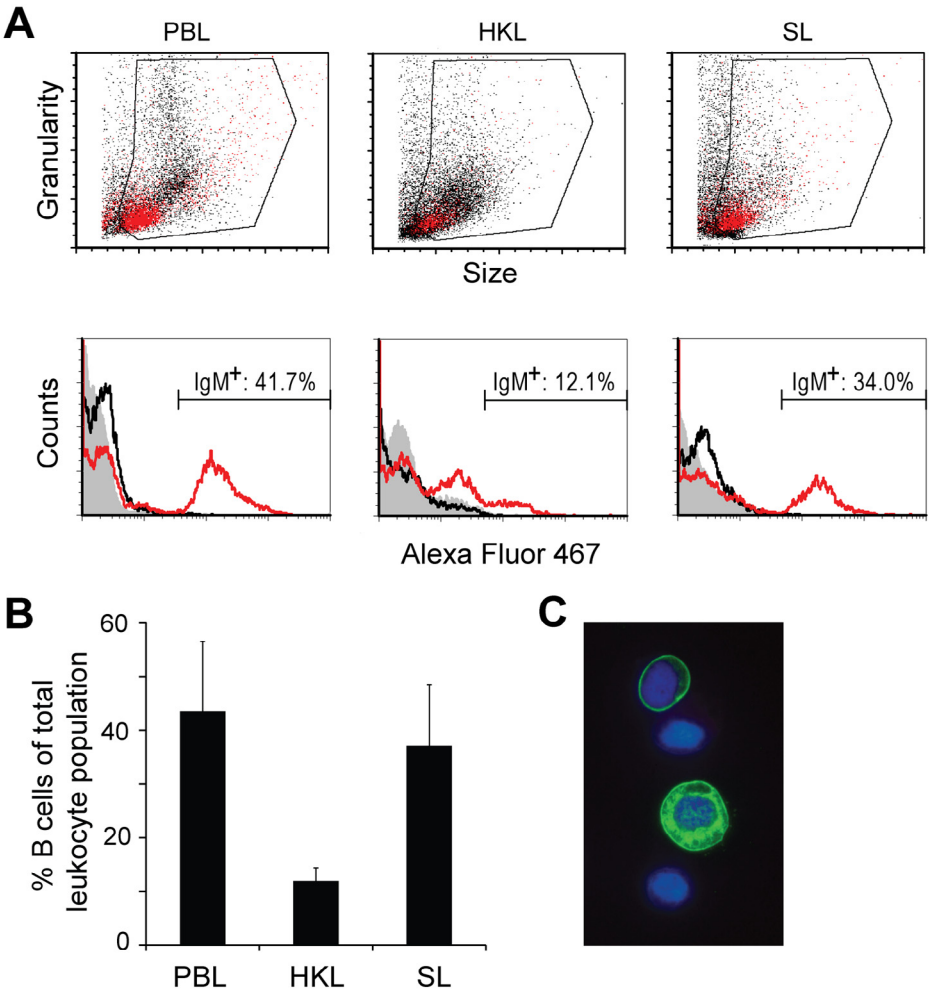

Fig. 2. Flow cytometry analyses and immunostaining of isolated leukocytes using anti-lumpfish IgM polyclonal serum. (**A**) Representative scatter plot of isolated leukocytes from peripheral blood (PBL), head kidney (HKL) and spleen (SL) stained with rabbit anti-lumpfish IgM serum (*upper panels*) and the corresponding histograms (*lower panels*). In the scatter plots, which distribute cells according to granularity and size, IgM⁺ cells are visualized as red dots. The gates define live cells. In the histograms, IgM⁺ cells, labeled with Alexa Fluor 467, are defined by the cursors. Red line = samples labeled with anti-IgM serum, black line = samples incubated with pre-serum and gray filled histograms are samples incubated with conjugate only (no primary antibody). (**B**) Diagram showing the percentages of IgM⁺ B cells of the total leukocyte populations PBL ($n = 8$), HKL ($n = 8$) and SL ($n = 8$). Means ± S.D. are shown. (**C**) Immunostaining using anti-IgM serum and Alexa Fluor 488 (IgM⁺ cells are green). Nuclei are stained blue with DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shown as red dots in the scatterplots in Fig. 2A, were found in the lymphocyte region consisting of small, agranular cells (Fig. 2A, *upper panels*). Corresponding histograms are shown in Fig. 2A, *lower panels*. The fraction of IgM⁺ B cells among PBL was 43.5 ± 13%, HKL 12.0 ± 2.5% and SL 37.1 ± 11.4% (Fig. 2B). The anti-IgM serum reacted with both small and large B cells as shown by immunofluorescence staining of cytospun leukocytes (Fig. 2C).

3.3. IgM⁺ B cells have high phagocytic activity

As a first step to characterize IgM⁺ B cells in lumpfish functionally, a phagocytosis assay was performed. PBL and HKL were incubated with 1 μm green fluorescent beads, labeled with the rabbit anti-lumpfish IgM serum and analyzed in a flow cytometer. A representative PBL sample is shown in Fig. 3A. A set of controls were included in the assay for each sample; PBL incubated with pre-serum without beads (quadrant *i*), PBL labeled with anti-IgM serum without beads (quadrant *ii*) and PBL incubated with pre-serum with beads (quadrant *iii*). Based on these controls, the IgM⁺ phagocytes were defined (quadrant *iv*, gate 7). The IgM⁺ B cells were found in the lymphoid region in the scatter plot (shown as red dots in Fig. 3A, gate 5), while the majority of the IgM⁻ cells among PBL were small agranular cells (shown as green dots in Fig. 3A, gate 6). The IgM⁺ phagocytes, shown in gate 7, had high phagocytic activity. Surprisingly, some small IgM⁻ cells (green dots, gate 8) had potent phagocytic ability with greater phagocytic capacity than the IgM⁺ B cells. The identity of the small IgM⁻ cells is currently not known.

A similar analysis of IgM⁺ phagocytic cells among HKL is shown in Fig. 3B. IgM⁺ B cells, shown as red dots in Fig. 3B, gates 5 and 7, were present in the lymphoid region, while the IgM⁻ cells, shown as green dots in Fig. 3B, gates 6 and 8, were present throughout the scatterplot, representing cells within both the lymphoid and granuloid/macrophage regions.

The IgM⁺ cells were the dominant phagocytic cells among PBL constituting 65.1 ± 11% of total phagocytic cells ($P < 0.0001$), while among HKL, the percentage of IgM⁺ B cells (9.8 ± 1.7%) of total phagocytic cells was significantly lower than other phagocytes ($P < 0.0001$) (Fig. 3C). The average percentage of phagocytic B cells of total B cells was, however, lower among PBL than HKL, 43.2 ± 16.8% and 56.9 ± 11.7%, respectively (Fig. 3D). This was also observed for the IgM⁻ cells. The percentages of phagocytic IgM⁻ cells of total IgM⁻ cells were lower among PBL than HKL, 25.1 ± 18.6% and 38.9 ± 9.5%, respectively. The analysis also showed that the percentages of IgM⁻ phagocytes among total IgM⁻ cells were significantly lower than IgM⁺

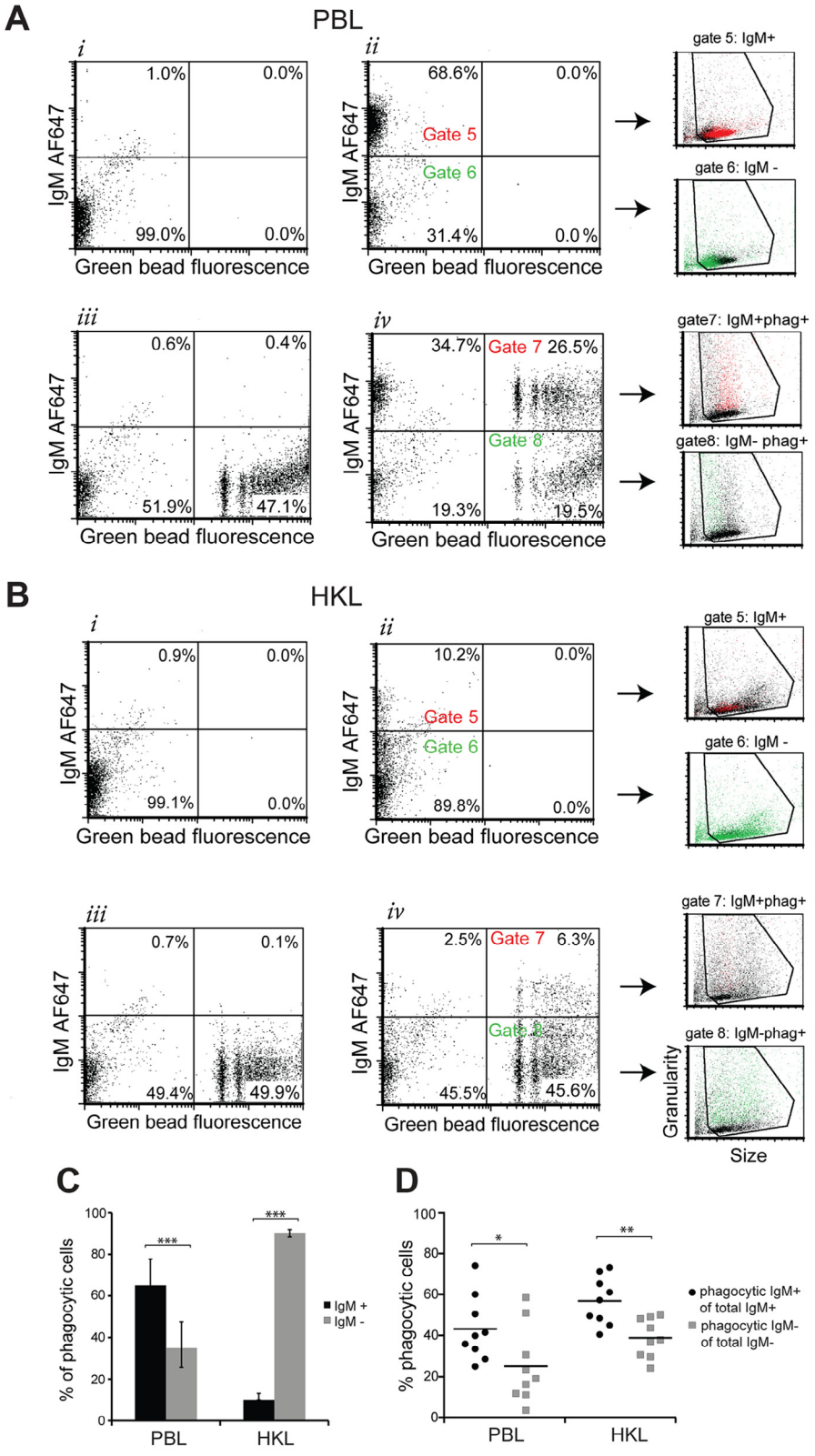

Fig. 3. IgM⁺ cells have phagocytic activity. Isolated leukocytes were incubated with 1 μm yellow green fluorescent beads at a cell:bead ratio of 1:20 and incubated at 15 °C for 5 hours. (**A**) Representative PBL and (**B**) HKL sample. *Panel i*: Quadrant plot of leukocytes incubated without green fluorescent beads and with pre-serum (negative control). *Panel ii*: Quadrant plot of leukocytes incubated with anti-IgM serum without beads. The IgM⁺ (gate 5, red dots) and IgM⁻ cells (gate 6, green dots) are shown in the scatterplots to the right. *Panel iii*: Quadrant plot of leukocytes incubated with pre-serum in the presence of beads. *Panel iv*: quadrant plots of leukocytes incubated with anti-IgM serum and beads. Phagocytic IgM⁺ cells (gate 7) are shown as red dots in the scatterplot to the left while the IgM⁻ phagocytes (gate 8) are shown as green dots. AF647 = Alexa Fluor 647. (**C**) Diagram with the percentages of IgM⁺ (black bars) and IgM⁻ phagocytes (gray bars) of the total number of phagocytic cells among PBL ($n = 9$) and HKL ($n = 9$). Means ± S.D. are shown. (**D**) The percentage of phagocytic IgM⁺ positive cells of total IgM⁺ population (black dots) and the fraction of IgM⁻ phagocytes among the total IgM⁻ leukocytes (gray squares) among PBL and HKL. Means are shown. Statistical significance: $P > 0.05$ = n.s., *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. The experiment was performed three times with different batches of fish. All experiments gave similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phagocytes among total IgM$^+$ cells in both PBL (P = 0.040) and HKL (P = 0.003) (Fig. 3D).

3.4. IgM$^+$ cells have high phagocytic ability

To compare the phagocytic capacity of IgM$^+$ B cells with other phagocytes, we investigated the cells' ability to ingest 1, 2, 3–8 and 9+ beads (Fig. 4). Unexpectedly, the PBL samples from the analyzed fish were clearly grouped into two groups, PBL group 1 (PBL1) containing leukocyte samples with a significant number of granulocytes (Fig. 4A) and group 2 (PBL2) where the granulocytes were low in numbers and the majority of the cells were small agranular cells (Fig. 4B). IgM$^+$ B cells in both PBL groups had a high phagocytic capacity (Fig. 4D, *left panel*). The majority of the B cells had ingested 3–8 beads and the percentages of IgM$^+$ B cells had phagocytized more than 3–8 beads were significantly higher than phagocytes with only 1 bead (P = 0.001 for PBL 1 and P = 0.0001 for PBL2). Among HKL, a significantly higher percentage of the IgM$^+$ B cells had ingested only 1 bead compared to IgM$^+$ B cells in blood (P = 0.024 in PBL1 and P < 0.001 in PBL2). The fraction of IgM$^+$ B cells with high capacity (9 or more beads) was significantly lower among HKL IgM$^+$ B cells than IgM$^+$ B cells in blood (P = 0.021 in PBL1 and P = 0.001 in PBL2) (Fig. 4D *left diagram*). Different from PBL, the percentage of IgM$^+$ cells among HKL with 9 or more beads was significantly lower (P = 0.0001) than those with 1 bead. The IgM$^-$ cells and their phagocytic capacity were different between PBL group 1 and group 2 (Fig. 4D, *right diagram*). The IgM$^-$ cells in PBL2 consisted mainly of small agranular cells (Fig. 4B) with remarkable high phagocytic capacity despite their small size. Most of the cells had ingested more than 3 beads and some of them could have more than 50 beads as the cell pictured in Fig. 4B. In PBL2, but not in PBL1, the percentage of cells with 9 or more beads was significantly higher than those with 3–8 beads (P < 0.0001). The phagocytic IgM$^-$ cells in PBL1 were granulocytes with lower phagocytic capacity (with 1 or 2 beads, and only a few had more than 5 ingested beads). Among PBL, the cell types with highest phagocytic capacity among the IgM$^-$ cells were the small agranular cells and monocytes. Similar to IgM$^+$ cells in HKL, the percentages of IgM$^-$ cells that have ingested 9 or more beads were significantly lower than those with 1 bead (p = 0.003) and 3–8 beads (p = 0.0001). Among IgM$^-$ cells in HKL, macrophages had the highest capacity to ingest beads (Fig. 4C). Phagocytic cells were cytospun and stained with colorrapid to visualize cell morphology (Fig. 4A, B, C).

3.5. Phenotypic characterization of sorted IgM$^+$ B cells

To further characterize the IgM$^+$ B cells in lumpfish, PBL were sorted into IgM$^+$ B cells, small IgM$^-$ cells and large IgM$^-$ cells (Fig. 5A). Post-sort analyses showed that the IgM$^+$ cells were isolated to a purity of 87–92%. The IgM$^+$ cells stained negatively for myeloperoxidase (MPO), but were positive for acid phosphatase (AcP) (Fig. 5B). The small IgM$^-$ cells were mononuclear leukocytes of lymphocyte size or smaller and stained negative for MPO similar to the IgM$^+$ cells. The smallest cells in this fraction stained most intensely for AcP (Figs. 5B and 6) which indicate the presence of a high number enzymes and/or high enzymatic activity. The large IgM$^-$ cells consisted mainly of neutrophilic granulocytes, but monocytes were also present. The neutrophils stained positively for MPO while the monocytes were negative. Both cell types stained positive for AcP (Fig. 5B). To investigate the ultrastructure of the sorted cells, the different fractions were prepared for TEM (Fig. 5C). The IgM$^+$ cells contained a few cytoplasmic vacuoles while vacuoles were more abundant in the small IgM$^-$ cells. Among the large IgM$^-$ cells were the neutrophils containing different types of granules and monocytes with internal vesicles and ribosomes in the cytoplasm.

For a more detailed comparison with regards to morphology and cytochemical staining of IgM$^+$ cells with other cell-types observed among isolated leukocytes, representative single cells of different subtypes are shown in Fig. 6. Among the IgM$^-$ cell-types we identified cells with high nucleus to cytoplasm ratio, similar to the B cells, that are likely T cells and we observed three subsets of neutrophils with bilobed, bean-shaped and rounded nucleus. Many of the neutrophils contained a large cytoplasm and had irregular cell morphology. All the neutrophils were positive for MPO. Basophils were identified, but MPO staining of the basophils was not determined due to the low abundance of these cells. Monocytes, macrophages and cells resembling dendritic cells were observed. These cells were all negative for MPO (Fig. 6). The cell subsets with highest AcP activity were monocytes/macrophages and some of the smallest IgM$^-$ cells that might be precursor cells or immature cells. All cell types had tartrate resistant acid phosphatases.

3.6. Specific antibodies are produced upon immunization

Single radial immunodiffusion (SRID) assay was performed to determine the total IgM concentration in serum of normal fish and upon immunization. In small, non-treated fish the concentration was 0.140 ± 0.099 mg ml^{-1} (n = 27) (Supplementary Fig. S2).

The SRID assay was also applied to serum from fish immunized with different bacteria, 2 isolates of atypical *Aeromonas salmonicidae*, *Vibrio anguillarum* (also known as *Listonella*), *V. ordalii* and a *Pasturella*-like bacteria, all isolated from lumpfish. As shown in Fig. 7B, the level of total IgM in serum was not significantly higher in the immunized fish compared with the controls. However, the levels of specific antibodies, measured with ELISA, were significantly higher in the immunized fish, except those immunized with *V. ordalii* (Fig. 7D). The production of specific antibodies verifies adaptive immunity in lumpfish.

4. Discussion

We have characterized IgM$^+$ B cells in lumpfish and compared these B cells with other cell types morphologically, phenotypically and functionally. Analysis of serum immunoglobulin showed that it is a tetramer of IgM type, based on molecular weight analyses of non-reduced and reduced isolated immunoglobulin and rabbit anti-lumpfish IgM serum. The IgM isotype is the dominating serum immunoglobulin also in other fish species (Sunyer, 2013). The produced rabbit antiserum to lumpfish IgM reacted with both heavy and light chains of purified IgM and, although weakly, with bands of corresponding size in the PBL samples (Supplementary Fig. S1). The weak reaction with PBL might be because of the number of cells in the leukocyte sample loaded on the gel or amount of IgM per cell. Further, the antiserum reacted with leukocytes consistent with lymphocyte morphology and size as shown by flow cytometry, staining of sorted cells and by immunofluorescence. Accordingly, this antiserum could be used to identify and isolate IgM$^+$ cells.

The percentages of IgM$^+$ cells among PBL, HKL and SL are comparable to those found in other fish species like trout (Abos et al., 2013) and Atlantic salmon (Øverland et al., 2010; Pettersen et al., 2000; Supplementary Fig. S3). In Atlantic cod, the percentages of B cells among PBL are lower compared with those of salmonids and lumpfish, while the level of cod B cells among isolated leucocytes from spleen is higher in cod (Rønneseth et al., 2007). In humans, the main population among PBL is neutrophils being about 70% of the leukocytes, while in fish the IgM$^+$ B cells seem to be the dominating leukocyte population in blood. The immunological significance of the high levels of B cells among PBL in fish is not known, but the high level, in addition to their high phagocytic ability, suggests that B cells in fish are a major part of the teleost innate immune defense

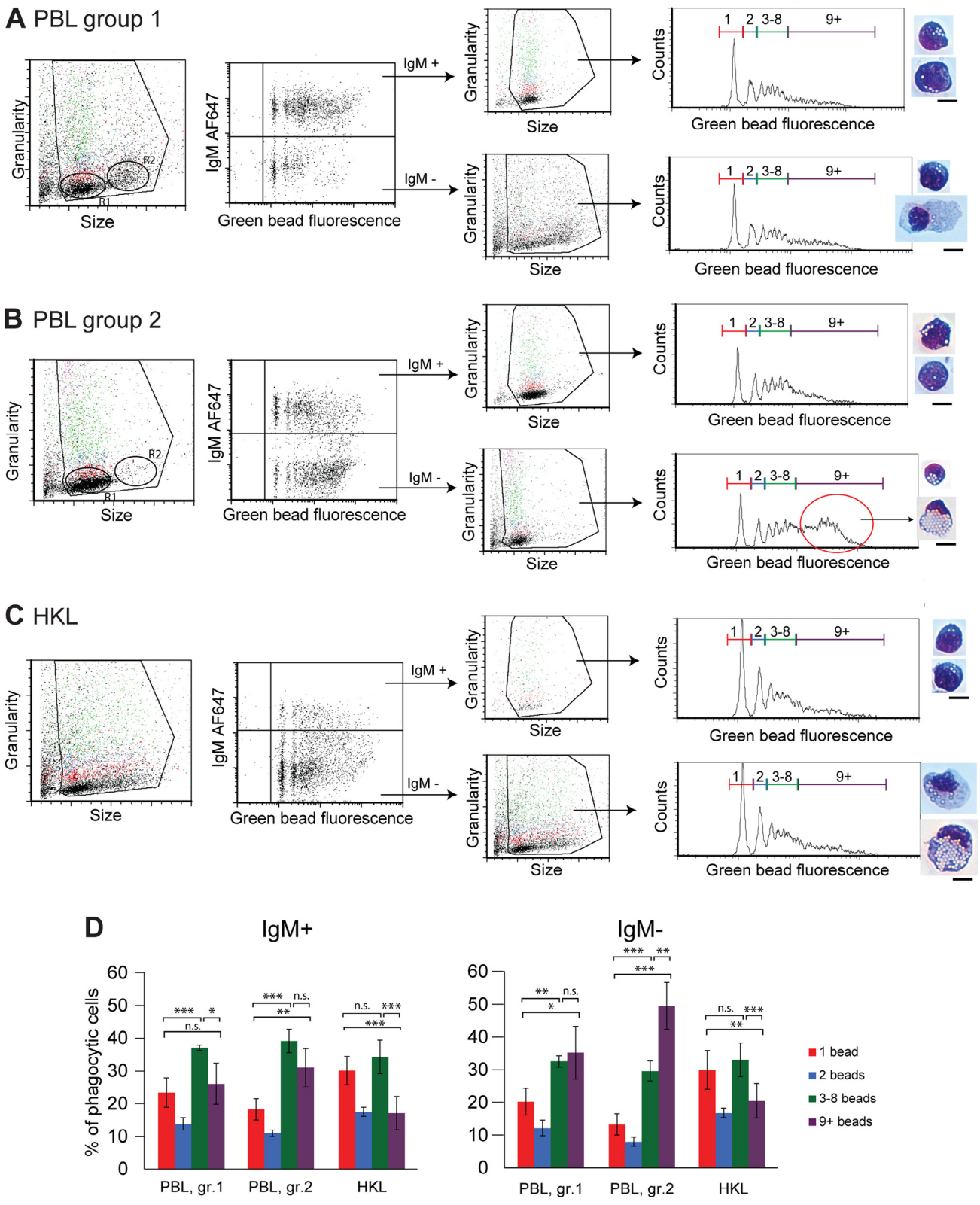

Fig. 4. IgM⁺ cells have high phagocytic capacity. Isolated leukocytes were incubated with 1 μm yellow green fluorescent beads at a cell:bead ratio of 1:20 and incubated at 15 °C for 5 hours. The PBL samples divided into group 1 with several neutrophils and group 2 with few neutrophils. (**A**) PBL, group 1 ($n = 4$) and (**B**) PBL, group 2 ($n = 4$) and (**C**) HKL ($n = 8$). *Left panels*: Scatterplots showing the phagocytic cells. R1 = lymphoid region, R2 = granuloid region. Red dots = cells with 1 bead, blue = cells with two beads, green = cells with 3–8 beads and purple = 9 or more beads. The gates define live cells. *Second left panels*: quadrant plots showing IgM⁺ and IgM⁻ phagocytes. AF647 = Alexa Fluor 647. *Second right panels*: Distribution of IgM⁺ and IgM⁻ phagocytes in scatter plots. *Right panels*: Histograms showing the number of beads ingested by phagocytic cells. The peaks define different numbers of beads ingested. Colorrapid staining of representative phagocytes are shown. Scale bar = 5 μm. The red circle in the histogram to the right in (B) show the IgM⁻ cells with extremely high phagocytic capacity despite their small size. (**D**) Diagrams of phagocytic IgM⁺ (left diagram) and IgM⁻ cells (right diagram) among PBL group 1, PBL group 2 and HKL. The colors of the bars represent various numbers of beads as indicated in the key. Means ± S.D. are shown. Statistical significance: P > 0.05 = n.s., *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

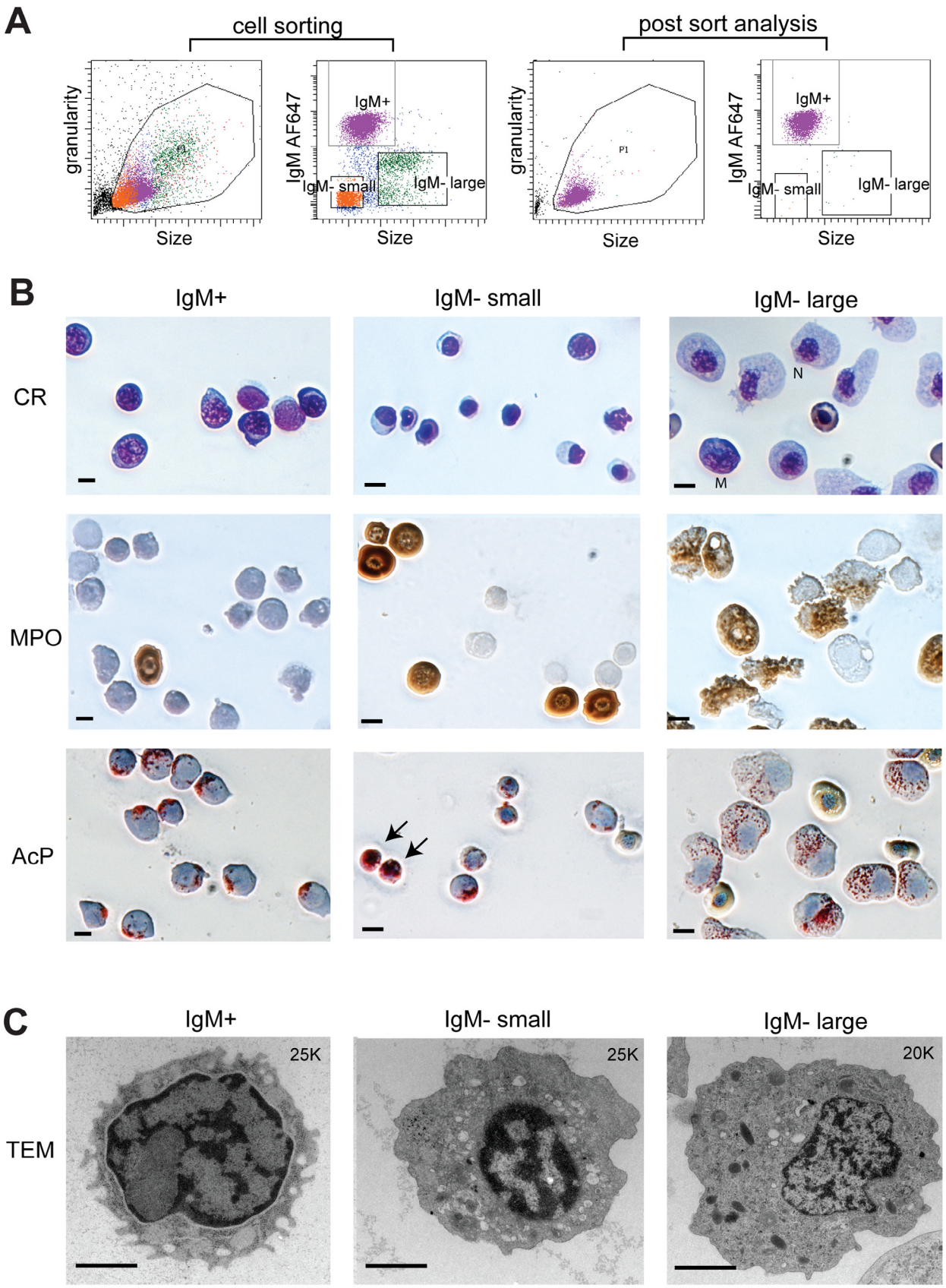

Fig. 5. Cytochemical staining and ultrastructural analyses of IgM⁺ cells, small IgM⁻ cells and large IgM⁻ sorted from PBL. (**A**) Cell sorting and post sort analysis. *Left panel*: Scatterplot of a representative PBL sample. The gate defines live single cells. *Second left panel*: Scatterplot showing IgM⁺ AF647 versus size. The squares define sorted cells; IgM⁺ cells, small IgM⁻ cells and large IgM⁻ cells. The two plots to the right show post sort analysis of IgM⁺ cells. (**B**) Colorrapid (CR) and cytochemical staining of sorted cells. MPO = myeloperoxidase, AcP = acid phosphatase, N = neutrophil and M = monocytes. Note that RBC stain positive in the MPO staining and thus function as a positive control for the staining procedure. Scale bar = 5 μm. (**C**) Ultrastructure of IgM⁺ (*left picture*), IgM⁻ small (*middle picture*) and IgM⁻ large cells (*right picture*). Representative cells are shown. The magnification are shown as numbers in the upper right corner of each picture, K = 1000. Scale bar = 1 μm.

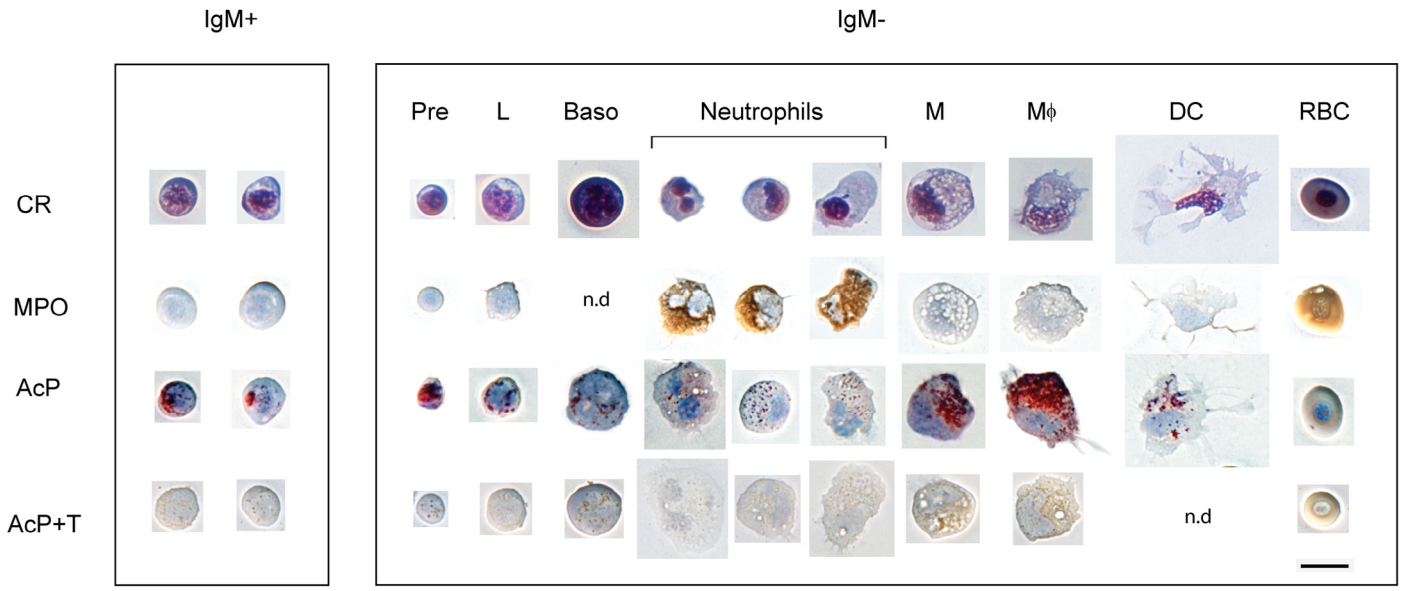

Fig. 6. Morphologic and phenotypic comparison of IgM⁺ cells with different cell types. CR = colorrapid, MPO = myeloperoxidase, AcP = acid phosphatase, AcP + T = acid phosphatase + tartrate, Pre = possible precursor cells, L = lymphocyte, Baso = basophil, neutrophils include cells with bilobed, kidney shaped and rounded nuclei, M = monocytes, Mϕ = macrophage, DC = dendritic-like cell, RBC = red blood cell. n.d. = not determined (due to the low number/absence of cells observed). Pictures are captured with 63× objective and representative cells are shown. Scale bar 10 μm.

mechanisms. In this study we have identified and characterized the lumpfish IgM⁺ B cell linage. Other immunoglobulin isotypes are not yet identified in lumpfish, although IgT⁺ cells and free IgT immunoglobulin, which are shown to be important for mucosal immunity in other fish species like trout (Castro et al., 2013; Zhang et al., 2010), may be present in this species.

For analyses of lumpfish B cell functions, we performed a phagocytosis assay using fluorescent beads. The flow cytometry analyses showed that IgM⁺ B cells in lumpfish had high phagocytic ability and capacity. Among PBL, the IgM⁺ cells constitute the main fraction of the phagocytic cells (65.1 ± 11%), while the IgM⁺ cells isolated from head kidney constituted only about 10% of the phagocytes. The high difference between the proportions of IgM⁺ phagocytes of the total phagocytic population in PBL and HKL is similar to trout (Abos et al., 2013) and expected due to the different abundance of IgM⁺ B cells among the isolated cells. Although the fraction of IgM⁺ phagocytes of total IgM⁺ cells in PBL was highly variable, varied from 25 to 74%, in average, the fraction of phagocytic IgM⁺ cells is higher among HKL than PBL, 56.9 ± 11.7% and 43.2 ± 16.8%, respectively. Interestingly, the IgM⁺ cells in blood have higher phagocytic capacity than those isolated from head kidney, indicating that factors like maturation may affect cell function. The high phagocytic capacity of the IgM⁺ B cells with ability to ingest more than 9 beads was unexpected. Also, in a previous study, we reported that leukocytes isolated from large wild caught lumpfish have high phagocytic ability (Haugland et al., 2012a), suggesting that the innate immune mechanisms in lumpfish are very effective, involving phagocytes like monocytes/macrophages, small agranular cells, B cells and neutrophils.

A striking observation was that IgM⁻ cells of equal or smaller size as the IgM⁺ cells had very high phagocytic capacity. These small mononuclear cells, which had ingested numerous beads, were easily observed in flow cytometry analysis and colorrapid staining of phagocytic cells from PBL. The nature of these cells is currently not known, but similar cells have been reported from trout (Li et al., 2006), Atlantic salmon (Haugland et al., 2012b) and wrasse (Haugland et al., 2014). We have earlier suggested for salmon and wrasse that these cells may be precursor cells or immature cells (Haugland et al., 2012a, 2014). They stain strongly positive for AcP and they may possibly belong to the myeloid linage, be a DC or B cell precursor. If they are B cell precursors this can support a relation between B cells and myeloid cells as suggested previously (Li et al., 2006). As phagocytic activity of B cells also have been reported from the evolutionary distant teleost species, rainbow trout and Atlantic salmon (order *Salmoniformes*), Atlantic cod (order *Gadiformes*) and catfish (order *Siluriformes*). The ability of B cells to be highly phagocytic seems to be a rather general feature of evolutionary distant teleosts, but variations exist as zebrafish IgM⁺ B cells were not particularly phagocytic (Parra et al., 2012). One must, however, keep in mind that about 40,000 known fish species represent a considerable phylogenetic diversity. The current knowledge showing similarities and differences in immunity within the teleosts and to mammals are based on studies of a few fish species and new findings extending the present knowledge are expected as more species are investigated. Phagocytic B cells have also been reported from amphibians (Li et al., 2006) and reptiles (Zimmerman et al., 2010). In addition, in mice, phagocytic B-1 cell subset has now been identified and was found to dominate in the peritoneal cavity. The phagocytosis was performed independent of the B cell receptor (BCR), but the cells presented antigen to CD4⁺ cells (Gao et al., 2012; Parra et al., 2012). Also mouse liver B cells have been shown to have high proportions of phagocytic B cells compared to the spleen cells (Nakashima et al., 2012). Both the fish phagocytic B cells and the B-1 cells have microbicidal abilities (reviewed in Sunyer, 2012). It has been hypothesized that the fish phagocytic B cells and the mammalian B-1 cells may be the most evolutionarily ancient B cell linage (Sunyer, 2013). It is well-known that fish B cells have MHC II and thus ability to present antigens, however, the mechanisms underlying the ability of fish B cells to present antigens to T cells is not clear. As there are teleost species, like Atlantic cod (Pilstrøm et al., 2005; Star et al., 2011), lacking MHC II, it will be interesting to investigate whether lumpfish have MHCII and if so, its presence on B cells.

To further investigate the B cell function, we performed cytochemical staining of sorted cells, which give valuable information about enzymes involved in degradation of microbes. One such enzyme is MPO which is part of an oxygen-dependent killing

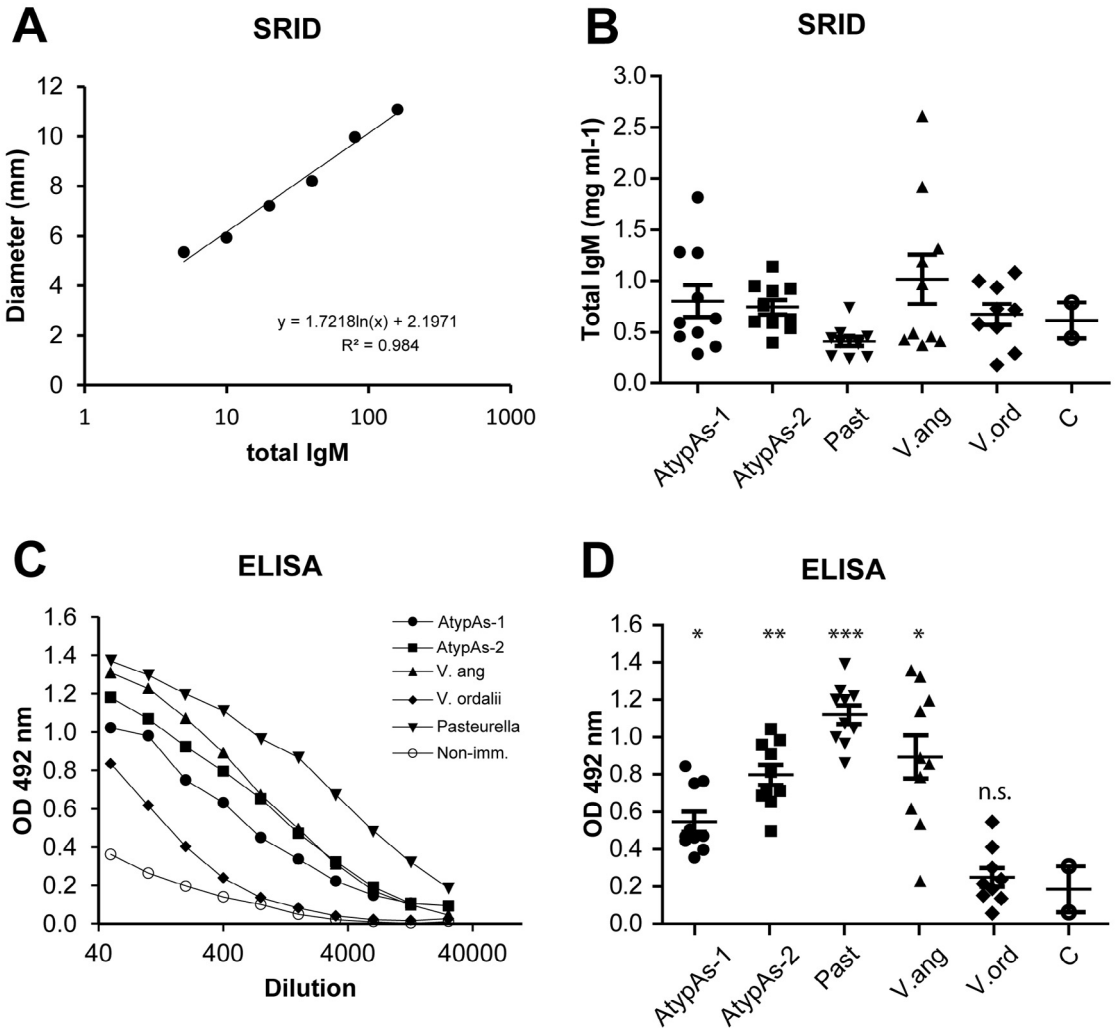

Fig. 7. Determination of total IgM and level of specific antibodies upon immunization determined by SRID and ELISA. (**A**) SRID standard curve of purified IgM. (**B**) Diagram showing total [IgM] in serum (mg ml^{-1}) in fish immunized with bacteria measured with the SRID method. AtypAs = atypical *Aeromonas salmonicida*, two different isolates, Past = *Pasteurella*-like bacteria, V. ang = *Vibrio anguillarum*, V. ord = *V. ordalii*, C = non-immunized controls. Average with standard deviation is shown. (**C**) ELISA. Dilution curve of sera from fish immunized with bacteria (each spot is the average value from the 10 fish within each group, except for the non-immunized controls were $n = 2$). (**D**) ELISA diagram showing the individual measurements within each group at serum dilution 1:400. Statistical significance: $P > 0.05$ = n.s., *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

mechanism. We found that IgM^+ B cells stain negatively for this enzyme. This is similar to B cells in other teleosts and mammals. It has to be noted that RBC stain positive in the MPO procedure. The only MPO positive leukocytes were neutrophils observed in fraction of large IgM^- sorted cells. The monocytes/macrophages were negative for MPO.

We have earlier shown that innate immune mechanisms of lumpfish are efficient (Haugland et al., 2012a), but knowledge of the lumpfish's adaptive immunity is largely unexplored. Therefore, as a first step, we measured the concentration of total IgM in serum and determined the presence of specific antigens in immunized fish. Although there were no significant differences in total IgM level upon immunization, the presence of specific antibodies was significantly higher in the immunized fish (with the exception of those immunized with *V. ordalii*). The result is as expected since the specific antibodies constitute only a minor fraction of the total IgM. Production of specific antibodies was measured by ELISA verifying adaptive immunity, but, further studies are needed to identify the magnitude of the adaptive immune-responses in lumpfish with regard to the protective immunity to pathogens upon vaccination and to fully understand the underlying mechanisms.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2015.05.010.

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