Sains Malaysiana 52(8)(2023): 2163-2173
http://doi.org/10.17576/jsm-2023-5208-01

Oil Based Inactivated Vaccine Formulation for Furunculosis (*A. salmonicida*) and Protective Immune Response of Rainbow Trout and Brown Trout

(Formulasi Vaksin Tidak Diaktifkan Berasaskan Minyak untuk Furunkulosis (*A. salmonicida*) dan Tindak Balas Imun Pelindung bagi Trout Pelangi dan Trout Perang)

Muhammad Akram[1], Muhammad Hafeez-Ur-Rehman[1*], Farzana Abbas[1], Imran Altaf[2], Sidra Kanwal[3], Kashif Ali[4], Nabila Gulzar[5], Muhammad Ajmal[5], Muhammad Asad[4] & Javaid Iqbal[6]

[1]*University of Veterinary and Animal Sciences, Department of Fisheries and Aquaculture, Lahore, Punjab, Pakistan*
[2]*University of Veterinary and Animal Sciences, Department of Microbiology, Lahore, Punjab, Pakistan*
[3]*Department of Zoology, University of Okara, Pakistan*
[4]*Department of Zoology, Division of Science & Technology, University of Education, Lahore, Pakistan*
[5]*Department of Dairy Technology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[6]*Institute of Zoology, Bahauddin Zakariya University, Multan*

Received: 31 January 2023/Accepted: 26 July 2023

ABSTRACT

The development and growth of the fisheries and aquaculture industries are significantly hampered by illnesses. It is critical to combat pathogenic illnesses, especially bacterial ones. Furunculosis in salmon is mostly brought on by *Aeromonas salmonicida* in rainbow and brown trout. To control this pathogen, vaccines have been identified as a significant tool. In the present study, we have formulated an inactivated vaccine with oil as an adjuvant and estimated its efficacy. The lethal dose of ArS-Pak-19, was calculated and injected intraperitoneally to the fishes. To analyze the infection, samples of kidney, liver, spleen, and blood were collected at specific times. To estimate the immunogenicity of the vaccine, an experiment was designed. One hundred sixty fishes were distributed into 8 tanks including, six experimental groups and two control groups with its replicates, vaccines injected intraperitoneally 1.6×10^7, 1.6×10^8, and 1.6×10^9 and blood samples were taken fortnightly for 56 days to calculate the antibodies titers. After immunization these groups were challenged with *Aeromonas salmonicida* (ArS-Pak-19) intraperitoneally. At 7th day of post infection, it appeared in the liver, spleen, and kidney. The relative percentage of survival was estimated with control groups at 30 days after challenge. The relative percentage of survival was 80%. The IgM titers were higher at 24 days of post immunization. We also analyzed that antibodies non-specifically bound with the A-layer of *Aeromonas salmonicida.* The findings of this study offer evidence that vaccinations boost fishes immunity and serve as a roadmap to further vaccination initiatives.

Keywords: Antibodies; IgM titres; immunization; pathogen; pathogenicity

ABSTRAK

Pembangunan dan pertumbuhan industri perikanan dan akuakultur terjejas dengan ketara oleh penyakit. Ia adalah penting untuk memerangi penyakit patogen, terutamanya bakteria. Furunkulosis pada salmon kebanyakannya disebabkan oleh *Aeromonas salmonicida* pada trout pelangi dan coklat perang. Untuk mengawal patogen ini, vaksin telah dikenal pasti sebagai alat penting. Dalam kajian ini, kami telah merumuskan vaksin yang tidak aktif dengan minyak sebagai pembantu dan menganggarkan keberkesanannya. Dos maut ArS-Pak-19 telah dihitung dan disuntik secara intraperitoneum kepada ikan. Untuk menganalisis jangkitan, sampel buah pinggang, hati, limpa dan darah dikumpulkan pada masa tertentu. Untuk menganggarkan keimunogeman vaksin, satu uji kaji telah direka. Satu ratus enam puluh ikan telah diagihkan ke dalam 8 tangki termasuk, enam kumpulan uji kaji dan dua kumpulan kawalan dengan replikasinya, vaksin yang disuntik secara intraperitoneum 1.6×10^7, 1.6×10^8 dan 1.6×10^9 dan sampel darah diambil dua minggu sekali selama 56 hari untuk menghitung titer antibodi. Selepas imunisasi kumpulan ini dicabar

dengan *Aeromonas salmonicida* (ArS-Pak-19) secara intraperitoneum. Pada hari ke-7 selepas jangkitan, ia muncul di hati, limpa dan buah pinggang. Peratusan relatif kemandirian dianggarkan dengan kumpulan kawalan pada 30 hari selepas cabaran. Peratusan relatif kemandirian ialah 80%. Titer IgM lebih tinggi pada 24 hari selepas imunisasi. Kami juga menganalisis bahawa antibodi tidak terikat secara khusus dengan lapisan-A *Aeromonas salmonicida*. Penemuan kajian ini menawarkan bukti bahawa vaksinasi meningkatkan imuniti ikan dan berfungsi sebagai peta jalan kepada inisiatif vaksinasi selanjutnya.

Kata kunci: Antibodi; titer IgM; imunisasi; patogen; sifat patogen

Introduction

At present there are several hazardous concerns shown in aquaculture, which are environmental degradation and outbreaks of diseases. These factors put the aquaculture industry in danger and by a large number of mortalities, effects on reproduction ability and also have influence on the efficiency of feed conversion ratio. The FAO estimates that 62 disease outbreaks in aquaculture result in a yearly loss of around 6 billion US dollars (Brummett 2014). Although the diseases have significant economic impact on aquaculture particularly in Asia, it is still not analyzed scientifically due to insufficient information about the mortality caused by diseases. Some bacteria have been causing some major disease in the aquaculture such as *Edwardsiella* species, *Flavobacterium* species and *Aeromonas* species. In farms on small scale, to control disease outbreaks, decontamination of farming systems might not be operated.

Bacterial and viral pathogens are very critical factors that hindered the growth of the fisheries and aquaculture. There is scarcity of information about the pathogen's life cycle, its interaction with the host to develop effective vaccines for fishes (Sommerset et al. 2005). Furunculosis caused by *A. salmonicida*, is a significant cause of mortality worldwide in the industry of fisheries and aquaculture particularly in salmonid, as was reported in 1890 (Janda & Abbott 2010). Owing to this, pathogen antibiotics are being used in the aquaculture industry at commercial level. Furunculosis present in the form of chronic or acute. In chronic infection, it has symptoms such as skin with furuncles, boils, lack of appetite, lethargy, and dark skin. While in the acute infection, the epidermis has necrotic lesions and septicemia followed by mortality (Austin & Austin 2016; Dallaire-Dufresne et al. 2014). Appearance of the symptoms of infection is owing to the secretory system (Type III) of the pathogen. This provides the bacteria with an ability to penetrate proteins (effector proteins) into the cells of the host tissues, followed by evasion in the immune system as there is inhibition of phagocytosis and intracellular killing mechanism (Fast et al. 2009) along with lipopolysaccharides with acyltransferase, cholesterol, and glycerophospholipids (Lee & Elis 1991). There exist inter- and intra-specific susceptibility differences among various species of salmonids. For instance, variability has been seen in furunculosis among *Oncorhynchus keta, Oncorhynchus kisutch, Oncorhynchus tshawytscha.* In *S. trutta,* (brown trout) *Salmo salar* (Atlantic salmon) and *Salvelinus fontinalis* (brook trout), infection rate is more as compared to other species (Holten-Andersen, Dalsgaard & Buchmann 2012). *A. salmonicida* infection is variable due to genetic response of the host as it is related to efficient survival rate. Following *A. salmonicida* infection, transcriptome and proteomic analyses showed that there are enough receptors to recognize the pathogen, proteins to regulate iron, and anti-inflammatory cytokines (Long et al. 2015). Results of hepatic transcriptome showed that vaccinated fishes have a decreased number of transcripts that intricate in the motility and conscription of immune cells, including integrin (binding proteins), annexins, and chemotaxin (Skugor et al. 2009). Resistant salmon have significant haemolytic activity and survival is associated with the Th2 responses (Zhang et al. 2011). Furthermore, survival is associated with genotypes or allelic variants in salmon (Croisetiere et al. 2008; Kjoglum et al. 2008). Furunculosis outbreaks are seen to occur in the farming operations. Salmons can tolerate a very low temperature due to high concentration of plasma electrolyte. For the last few years, the occurrence of furunculosis has been reduced by the use of vaccines, but protection against furunculosis is inconsistent and outbreaks of infection occur obstinately (Gudding & Van Muiswinkel 2013). Success of vaccines is limited due to lack of knowledge of pathogenicity. Since the last few years advancement has become evident in the field of genetic analysis, which has helped to understand the host pathogen relationship and pathogenicity. There are several studies which report

the use of these advances in the field of genetic analysis during infection (Sundvold et al. 2010). At present, there is not any data on the efficacy of the vaccine to control the furunculosis.

A. salmonicida is a notorious pathogen of various cultured fish species (Austin & Austin 2012; Cipriano & Bullock 2001). To control *A. salmonicida* in aquaculture, the use of a good vaccine is of great importance. It reduces the use of antibiotics and has effective control on diseases (Mahoney et al. 2007; Shoemaker et al. 2009; Santander et al. 2012). According to food security and food safety policies, antibiotics are not preferred to control diseases. Infectious diseases can be controlled to activate the immune system of the fish, as it is the most reliable and practical method (Magnadottir 2010). In the 1980's the salmon industry of Norway faced a huge loss due to furunculosis and vibriosis and successfully used vaccination to overcome these diseases (Sommerset et al. 2005). Effective vaccination has reduced the use of antibiotics and has increased production of the fisheries, but the use of vaccination in some parts of the world is still not practiced very well owing to some low value fish species and not having whole information regarding pathogen stimulated immune response and pathogenic agent. There are many fish vaccines registered for the salmonids in the USA, but in Southeast Asia, there are only a few vaccines for selective fish species.

Vaccines are considered an important tool to prevent infections and widely used in some parts of the world (Watts et al. 2017). Rainbow trout and Atlantic salmon aquaculture in Norway, USA and UK flourished in the 1980s followed by rapid expansion of disease, especially bacterial pathogens *A. salmonicida Yersinia ruckeri* and *Vibrio* species. To control such pathogenic infection, huge amounts of antibiotics were used; this leads to resistance in the bacteria. Vaccines were formulated for the first time at commercial level against Enteric Red Mouth, vibriosis and furunculosis. The first vaccine licensed in 1997 in the USA was against the Enteric Red Mouth disease in the salmonids (Gudding & Goodrich 2014). At present many small companies and 19 large companies make fish vaccines worldwide (Brudeseth et al. 2013). There are currently 24 commercially available vaccinations, up from two in the 1980s, when they were first utilized in fisheries and aquaculture (Adams & Subasinghe 2019; Shefat 2018). Vaccines are used for a large number of fish species: Tilapia, sea bream, yellow tail, rainbow trout and Atlantic salmon (Assefa & Abunna 2018). Most of the vaccines are whole cell, formalin killed vaccines, while in USA, live attenuated vaccines are also licensed and used for catfish (Klesius & Pridgeon 2014). Haematopoietic necrosis is treated by the use of DNA vaccine for Atlantic salmon in Canada (Alonso & Leong 2013). In Chile, recombinant vaccine is used to treat ISAV, the infectious salmon anemia virus, while subunit vaccine is used in Norway to treat haematopoietic necrosis. To use commercial vaccines there should be knowledge of nutrition, stress factors, life cycle, and immune system status. Most vaccines produced at commercial level have adjuvants and are injected intraperitoneally (Adams & Subasinghe 2019).

In Pakistan, there is no effective vaccine for furunculosis caused by *A. salmonicida.* Some countries use commercial vaccines against *A. salmonicida* such as AJM (Alpha Ject Micro®, Norway) for Atlantic salmon having weight 15 g or more (PHARMAQ 2020), FMIV® for salmonids weight 10 g or more, and Elanco Canada Limited has inactivated infectious agents with oil as adjuvant in liquid emulsion (Elanco Canada Limited 2020). In the USA a polyvalent vaccine was analyzed and found very successful for *A. salmonicida*, and bath vaccines are less effective than intraperitoneally injected vaccines (Arkoosh et al. 2018). The objective of this study was to evaluate the efficacy of an inactivated vaccine formulated with oil as an adjuvant in controlling furunculosis caused by *A. salmonicida* in rainbow and brown trout.

Materials and Methods

Preparation of Bacterins and Vaccine Formulation

A. salmonicida, ArS-Pak-19 (MW307221), ArS-Pak-GB1-19 (MW720959), ArS-Pak-MRE-19 (MW720960), ArS-Pak-SW2-19 (MW720961), ArS-SW1-Pak-19 (MW720962) (characterized, identified, and isolated in our other experiment: NCBI accession number MW307221)) were independently grown in the Trypticase Soy Broth with 100 µM, 2,2-dipyridyl at 20 ºC. These strains were washed with phosphate buffer saline (PBS, pH7.2), centrifuged at 4 ºC for 10 min, supplemented with 5% formalin and kept at 20 ºC for 72 h. By centrifugation at 4500 rpm, 4 ºC for 9 min, formalin was removed and bacterin was suspended in PBS. Cells were inactivated and dialyzed in one litter PBS and 5% formalin (3 times) at 4 ºC with continuous stirring. The inactivated cells were analyzed by streaking on growth media. The strains were mixed in equal amounts and

quantified by using bacterial counting technique and flow cytometry according to the instructions provided by the manufacturer. This bacterin was stored for further use at 4 ºC (Eslamloo et al. 2020).

Centrifugation at 4500 rpm for fifteen minutes was performed to collect the cells, after which they were washed three times with sterile phosphate buffer solution (PBS, 0.01 M, pH 7.0). The cell concentration was modified to 1×10^9 cells before the cells were re-suspended in sterile PBS. The mixture was kept chilled, at 4 ºC. The adjuvant Montanide ISA 201 VG (water-in-oil-in-water) emulsion was employed to increase the efficacy and lifespan of the vaccine. Vaccines were developed, method described by Sughra et al. (2021).

All the vaccines were properly homogenized and sterility was checked to verify vaccine safety following streaking on nutrient agar plate. Sterility of vaccines was verified by inoculating the vaccine in nutrient broth tubes for 3 days and incubating at 22 ºC.

DETERMINATION OF LETHAL DOSE IN (LD_{50}) OF *A. salmonicida* FOR RAINBOW TROUT

Rainbow trout and brown trout (20 each species) with average weight of 150±15 g were taken from Govt. Experimental Trout Hatchery, Kargha, Gilgit Baltistan and acclimatized in cemented tanks for 10 days. Fishes were fed on commercial diet (35%) once a day at 7 am. The LD_{50} of *A. salmonicida,* ArS-Pak-19 was estimated using standard protocols (Chakraborty et al. 2019). The fishes were anesthetized by MS 222 Syndel, Canada, and infected intraperitoneally (50 µL of bacterial) with three concentrations of bacteria (10^7, 10^8 and 10^9 Colony Forming Unit/mL). Out of 20, three fishes from each were monitored for mortality while 3 were used to re-isolate the pathogen to analyze the tissue colonization of *A. salmonicida.* LD_{50} was estimated by the following formula;

$$LD_{50} = 10^{a-(PD)}$$

where PD is the proportional distance, L% - 50%/ L%-H%; a is the $\log_{10}$ (mortality dilution factor >50%); H% is the (mortality dilution point >50%); and L% (mortality dilution point <50%) (Ramakrishnan 2016).

ANALYSIS OF TISSUES

Three fish from each species were captured and anesthetized by using "MS-222" and tissues were obtained from liver, kidney and spleen at 7 and 14 days of post injection, weighed and then standardized in PBS to form 1:10 fold dilution. Bacterial infestation was checked by using plate count method using MacConkey agar and Trypticase Soya Agar Congo red. These plates were incubated at 20 ºC for 72 h to estimate the Colony Forming Unit (CFU) of *A. salmonicida*, ArS-Pak-19. Sum of the bacteria was standardized to one gram of tissue as per initial tissue weight by this formula: CFU = (CFU count/Initial weight of the tissue) × 1 mL.

Sections of tissue were immobilized into 10% buffered formalin and placed for 72 hours at 25 ºC. Before block processing, formalin was removed and these samples were placed into 100% ethanol and went through standard histological procedure, Chandler and Robinson (2009). Tissues were stained with eosin and hematoxylin and analyzed under a compound microscope.

PROTOCOL FOR FISHES IMMUNIZATION

Rainbow trout and brown trout (n = 160, 20 fishes/tank) with an average weight of 150±15 g were acclimatized for 10 days and randomly stocked in 8 experimental groups; 6 vaccinated groups (3 for each species) and 2 control groups. Fishes were starved for 24 hours, and were immunized intraperitoneally by Vac A, (1.68×10^9 CFU/mL), Vac B (1.68×10^8 CFU/mL) and Vac C (1.68×10^7 CFU/mL) with 0.5 mL per dose of vaccine. In the controlled group, phosphate buffer saline was injected in equal quantities of each type of vaccine. Immunization was carried out using standard protocols. The length and weight of the fishes were measured fortnightly to analyze the SGR (specific growth rate) Blood samples from all groups were collected at intervals of 14 days within the non-lethal range.

After nine weeks of immunization, the weight of the fishes was measured. After one week of immunization, 10 fishes were subjected to the challenge study with one hundred times more than the lethal dose of ArS-Pak-19 and mortality was examined on a daily basis. By using the following formula, the RPS (relative percentage survival) was calculated; Relative percentage survival= (1- % Vaccinated mortality/% Saline mortality) × 100.

IMMUNOHISTOCHEMISTRY ANALYSIS

Spleen, liver, and kidney paraffin sections were used for the immunohistochemistry examination, which was performed on positively charged slides using rabbit IgG. Immunostainer used was automated to stain these sections of tissues with citrate-based antigen retrieval, at 100 ºC for 64 min, incubated for 32 min with rabbit IgG (negative control) and visualized using ultra view and hematoxylin counterstains. Anti-rainbow trout, anti-

brown trout and anti-*A. salmonicida*, *A. salmonicida,* anti-VapA, IgM from a rabbit previously exposed to VapA proteins from the outer membrane of *A. salmonicida* and IgM and IgY from chicken antibodies against rainbow trout (IgY chicken antibodies) conventionally produced (Somru BioSciences). The anti-rainbow trout, anti-brown trout and anti-*A. salmonicida* antibodies were used in a ratio of 1:500, and to detect IgM staining. Secondary antibody was applied with a ratio of 1:250 (alkaline phosphatase-conjugated anti-IgY) (Yang et al. 2019).

FLUORESCENCE VISUALIZATION AND CONFOCAL MICROSCOPY

A. salmonicida had an extracellular array of membranous proteins, which had affinities to bind with antibodies (Magnadottir et al. 2002). To estimate the specific immunoglobulins which bind with the A-layer of *A. salmonicida* confocal microscopy was used.

PURIFICATION OF IGM

Serum collected from blood samples was subjected to purification, the IgM through Mannan Binding Proteins (MBP) column kit, according to the protocols used for teleost with a little modification (Santander, Mitra & Curtiss III 2011). The purity and integrity of the fish IgM was analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and enumerated by UV & BCA (Bicinchoninic acid) quantification procedure (Sambrook & Russell 2001). High-quality IgM fractions were separated and purified. IgM was lyophilized and then suspended in Tris-HCl (20mM). These IgM was again subjected to the same procedure of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis.

ENZYME LINKED IMMUNOSORBENT ASSAY

It was estimated that the IgY and IgM will strongly bind with A-layer non-specifically. After immunization the titer of the IgM was analyzed with the help of direct enzyme-linked immunosorbent assay. From the serum sample of the fishes, the complement was inactivated to treat with temperature at 56 ºC for 30 min and fat from the serum was removed by centrifugation. After that, the supernatant was collected by centrifugation and stored at -80 ºC to determine the titer of IgM. 200 µL of this serum diluted in coating buffer (pH 9.8; 0.035 mM $NaHCO_3$; 0.015 mM Na_2CO_3) was transferred to 96 wells plates and incubated for 24 h. Then, the plate was washed three times with phosphate buffer saline and blocked with 150 µL blocking buffer (ChonBlock $_{TM}$) for 60 min at 37 ºC. The plate was washed three times with PBS and incubated with secondary antibody for 60 min at 37 ºC. Then, 100 µL of streptavidin HRP was added to the plate which was then incubated for 60 min at 37 ºC. Colour development was achieved by adding 120 µL of 1X TBM H_2O_2 followed by incubation in darkness for 30 min at 20-25 ºC. A 50 µL stop solution was added and optical density was measured at 450 nm. The curve was developed using standard protocols (Hnasko 2015).

STATISTICAL ANALYSIS

Collected data regarding antibodies and body weight were analyzed through one-way ANOVA technique using GLM procedure in SAS software (version 9.1). For comparison of significant treatment means Fisher's Least Significant Difference test was applied assuming significance level at $P \leq 0.05$. The following mathematical model was applied:

$$Y_{ij} = \mu + \tau_i + \epsilon_{ij}$$

where Y_{ij} is the Observation of dependent variable recorded on i^{th} treatment group; µ is the Population mean; τ_i = Effect of ith treatment group (i = 1, 2, 3); ϵ_{ij} is the Residual effect of jth observation on i^{th} treatment group, NID ~ 0, σ^2.

RESULTS

A. salmonicida INFECTION KINETICS AND CALCULATION OF LD_{50} IN RAINBOW TROUT

In three groups of the fishes, three doses of different values 10^9, 10^8, 10^7 of ArS-Pak-19 were given to calculate the LD $_{50}$. After 7 days of post infection, the symptoms of the infection were apparent. Within 8-10 days of post infection, 95% mortality was observed with 10^9 CFU/dose and 80% mortality was observed with 10^8 CFU/dose within 21 days of post infection. Mortality was less with 10^7 CFU/dose (70%) within 28 days. All these different groups were given the above mentioned doses to estimate bacterial colonization in various tissues from 5-10 days of post infection. At 5 days of post infection, there was no observed bacterial colonization with the lowest dose while at 10 days of post infection, bacterial colonization was observed.

EXPRESSION OF CD 10 AND IGM IN INFECTED FISHES TISSUES

To analyze the CD 10 and IgM in the tissues of the infected fishes, sections of the infected fish tissues

were collected at different days and processed for immunohistochemistry. At 10 days of post infection, there were several alterations observed in the tissues of the infected fishes such as tissue disorganization (Figure 1(A)-1(F)). Immunohistochemical analysis showed that at 10 days of post infection, there was an increase in the IgM in the kidney liver and spleen. CD 10 expression becomes less during infection.

IMMUNIZATION OF FISHES

One hundred and sixty fish were challenged with 9 weeks of post immunization with hundred times of the lethal dose. The mortality rate was recorded from eight to ten days after the challenge. The phosphate buffer saline group (controlled group) showed 85 % mortality, and the results of this were compared with the results of the vaccine to estimate the (RPS) relative percentage survival (Figure 2).

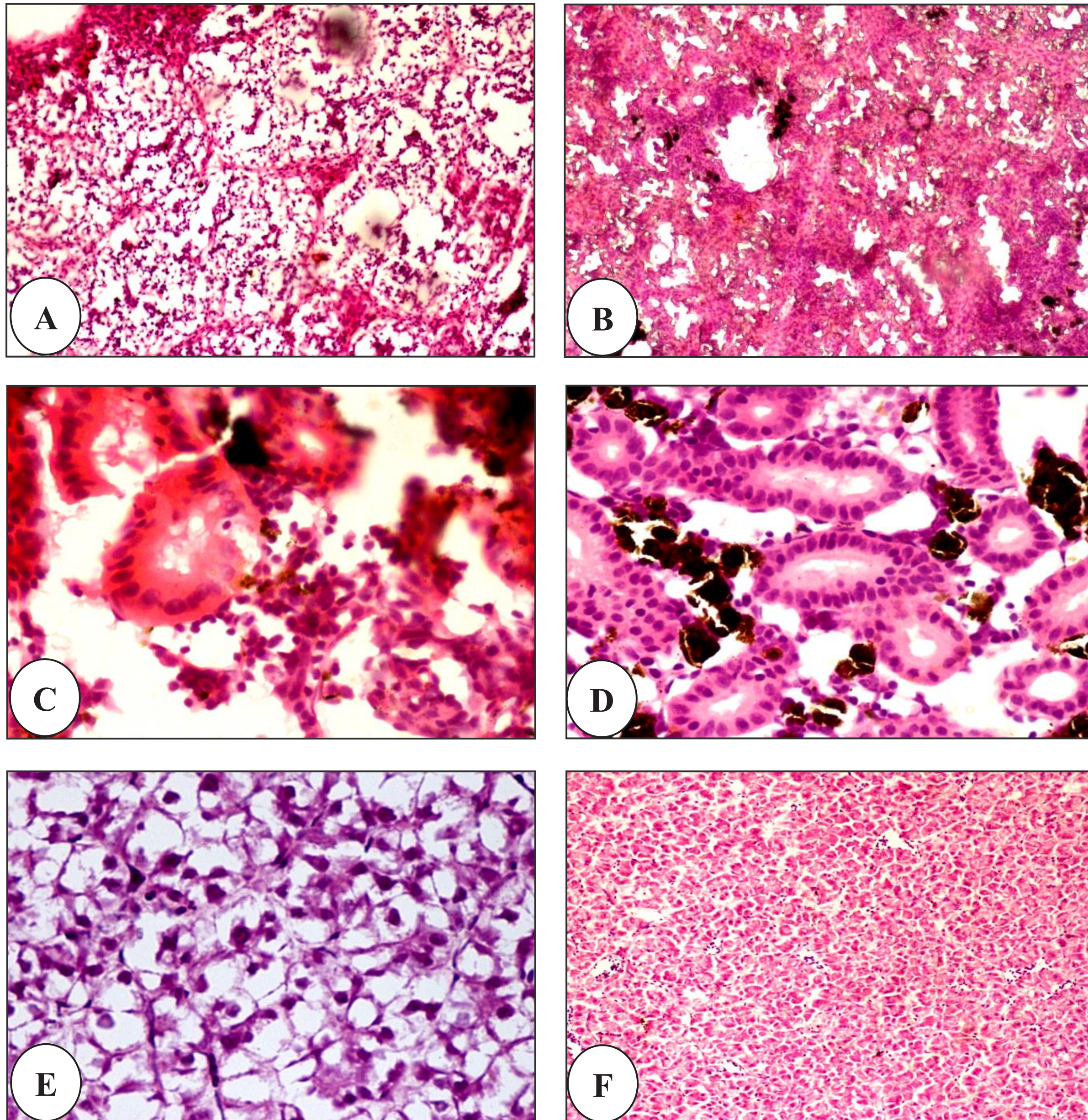

FIGURE 1. (A) Degenerative changes are apparent in the spleen of the fishes, (B) Normal spleen with no tissue changes were seen, (C) Peritubular infiltration of inflammatory cells is evident in the kidney of the fishes, (D) Normal kidney with no tissue changes were observed, (E) Hepatic cords are destroyed. Hepatocytes are swollen. Hydropic degeneration is seen in the hepatocytes. Many hepatocytes are undergoing necrosis, and (F) Normal liver tissues with no degenerative changes were observed and hepatic parenchyma were also normal (400×)

TITERS OF IGM IN IMMUNIZED FISHES

The IgM was refined from 200 mL serum, by using an MBP column kit. The IgM was lyophilized and obtained (IgM) 3,580 µg/mL. These antibodies were visualized with the help of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The heavy and light chains were about 75kDa and 25kDa, respectively (Mashoof & Crisitiello 2016). IgM titers against *A. salmonicida* were determined by indirect enzyme linked immunosorbent assay. The secondary antibody IgY non-specifically binds with *A. salmonicida*. A-layer influence of IgM titers against *A. salmonicida*. Different concentrations were prepared from 50-1.563 mg/mL to make a standard curve. By the use of natural logarithm, the concentrations of IgM were standardized. The concentration of IgM was estimated at 14, 28, 42, and 56 days after vaccination from non-lethal samples of the blood. The titers of IgM in the fishes were observed to be about 0.096 mg/mL. At 14 days of post immunization the IgM titers in the fish groups were elevated and observed a 0.201 mg/mL. At 28 days, the titers of IgM were estimated at 0.421 mg/mL. At 42 days of immunization, there was reduction in the IgM titers 0.402 mg/mL. At 56 days of post immunization, further reduction was observed in the IgM titers 0.325 mg/mL (Tables 1 & 2). Relationship between initial and final weight of the vaccinated fishes is given in Table 3.

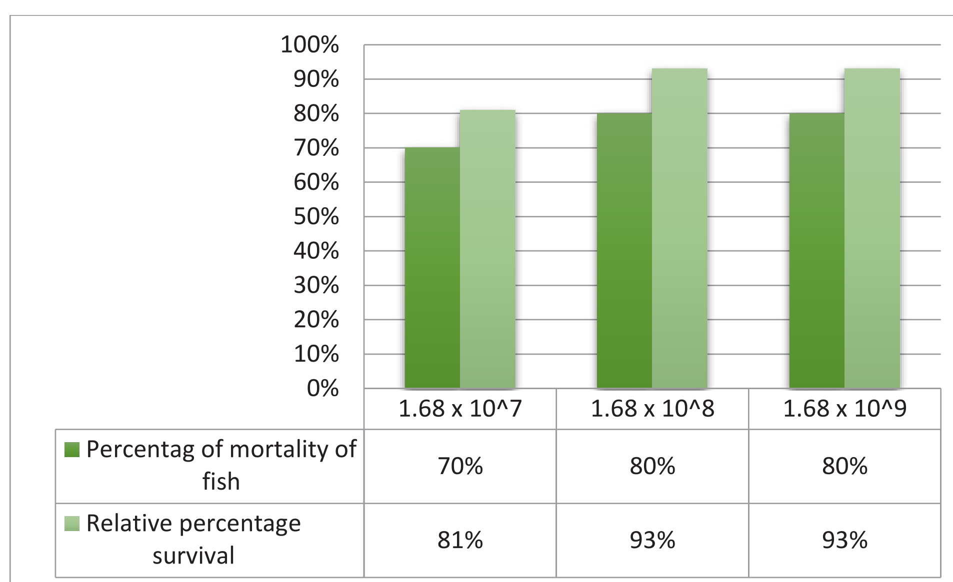

FIGURE 2. Percentage mortality and relative percentage survival of the fishes in response to the oil-based vaccine

TABLE 1. Challenge trials of oil adjuvanted vaccine against *A. salmonicida*, ArS-Pak-19, and antibodies response in rainbow trout

Treatment	Antibodies at (mg/mL)				
	0 day	14 day	28 day	42 day	56 day
1.68×10^7	0.09 ± 0.001	0.18^c ± 0.002	0.37^c ± 0.002	0.34^c ± 0.002	0.28^c ± 0.002
1.68×10^8	0.09 ± 0.001	0.19^b ± 0.001	0.39^b ± 0.002	0.37^b ± 0.001	0.33^a ± 0.002
1.68×10^9	0.09 ± 0.002	0.20^a ± 0.001	0.42^a ± 0.001	0.40^a ± 0.002	0.32^b ± 0.001
P-value	0.2903	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Superscripts on different means within column differ significantly at $P \leq 0.05$
Data were presented as lease square means ± standard errors
Data regarding growth and antibody response were analyzed through paired t-test, assuming significance level at $P \leq 0.05$; data were presented as least square mean ± standard errors

TABLE 2. Challenge trials of oil adjuvanted vaccine against *A. salmonicida*, ArS-Pak-19, and antibodies response in brown trout

Treatment	Antibodies at (mg/mL)				
	0 day	14 day	28 day	42 day	56 day
1.68×10^7	0.09 ± 0.001	0.18^c ± 0.002	0.38^c ± 0.002	0.34^c ± 0.002	0.28^c ± 0.003
1.68×10^8	0.09 ± 0.001	0.19^b ± 0.001	0.39^b ± 0.002	0.37^b ± 0.002	0.33^a ± 0.002
1.68×10^9	0.09 ± 0.001	0.20^a ± 0.001	0.42^a ± 0.001	0.40^a ± 0.002	0.32^b ± 0.002
P-value	0.3065	< 0.0001	< 0.0001	<0.0001	<0.0001

Superscripts on different means within column differ significantly at $P \leq 0.05$
Data were presented as least square means ± standard errors

TABLE 3. Relationship between initial and final weight of the vaccinated fishes

Treatment	Body weight (g)	
	Initial	Final
1.68×10^7	153.89 ± 1.31	260.96^c ± 1.66
1.68×10^8	150.73 ± 1.50	268.55^b ± 1.94
1.68×10^9	151.18 ± 1.32	274.76^a ± 1.17
P-value	0.2242	<0.0001

Superscripts on different means within column differ significantly at $P \leq 0.05$
Data were presented as lease square means ± standard errors

DISCUSSION

A. salmonicida is a causative agent of furunculosis in salmon, particularly in rainbow trout and transmitted in several other species (Krkosek 2017; Skrodenyte-Arbaciauskiene 2012). We have isolated ArS-Pak-19, from the infected rainbow trout and grew it *in vitro* (Leboffe & Pierce 2015). It was observed that *A. salmonicida* colonizes in the kidney and liver quickly. It is evident from the results that liver and kidney are the initial target sites. At 5 days of post infection, bacteremia was observed and infecting erythrocytes. It was also reported that *A. salmonicida* also colonized in the brain of rainbow trout (Valderrama et al. 2019). It is estimated erythrocytes might be the cause of transfer of it to the other body parts, recent knowledge also suggests the same. The mechanism of the protection of infectious diseases in fishes due to blood brain barrier is not clear. During infection of bacteria, encephalitis has been observed and there is no protection by blood brain barrier (Patterson et al. 2012; Pressley et al. 2005; Starliper 2011; Van Leeeuwen et al. 2014). Immunohistochemical analysis showed that after 10 days of infection, there was an increase in *A. salmonicida*. IgM is generated in fish by B lymphocytes, whereas CD 10 is a hematopoietic progenitor marker in the cells of the mammalian bone marrow (Parra, Takizawa & Sunyer 2013). In response to infection, there is an increase in IgM concentration, while in CD 10, a decrease was observed due to proliferation of B cells. Furunculosis symptoms were not apparent at the 5th day of infection with lowest dose while symptoms were evident when doses were higher. The results showed that *A. salmonicida* infection depends on the dose of ArS-Pak-19. We have formulated a vaccine using the following strains, *A. salmonicida,* ArS-Pak-19 (MW307221), ArS-Pak- GB1-19 (MW720959), ArS-Pak-MRE-19 (MW720960), ArS-Pak-SW2-19

(MW720961), ArS-SW1-Pak-19 (MW720962). The relative percentage survival was 80% which is more than the required percentage survival (Midtlyng 2016). To analyze the relative percentage survival of a vaccine, in a previous study, the relative percentage survival was at 81.7% (Arkoosh et al. 2018). This suggests similar results with our study. The analysis of bacterial load in non-immunized fish tissues was high. Some vaccinated fish groups also have a little colonization of bacteria, thus it is evident the vaccine still needs improvement. Furthermore, it is clear that VapA attaches antibodies in a non-specific way. This layer of *A. salmonicida* boosts the process of phagocytosis, the efficacy of this layer as a protective antigen is still not clear. This surface layer VapA has the same role as it is played by M protein in the *Streptococcus pyogens.* It is well characterized and has conserved serotype domains (Ling et al. 2019). Earlier studies have analyzed the titers of IgM and IgM (antigen specific) to estimate efficacy of the vaccine against *Flavobacteria columnare, Streptococcus agalactiae, Streptococcus iniae, Yerisina ruckeri, Vibrio anguillarum, A. salmonicida* and viruses (Brown et al. 2020; Hordvik 2015; Lulijwa et al. 2019; Mikkelsen et al. 2011; Ronneseth et al. 2015; Yin et al. 2019). In this study it is estimated that the total IgM is linked with the relative percentage survival of the fishes. We have also estimated the concentration serum with the relative concentration of the other components of the blood. On average, 25 g rainbow trout has 0.68 ± 0.67 mg/mL of serum (Castro et al. 2013). In our study it was estimated that the amount of serum was almost similar in the same size of fishes. The antibody titers from 160 mL - 210 mL was about 3.7 mg/mL (Kamil et al. 2011). Vaccinated fishes produced higher concentrations of antibodies; the IgM titers were higher at 28 days of post vaccination. At 42 days of post-vaccination, it starts to decline. In this study, the vaccine produced a significant immune response, and the results of our study are similar to earlier research where peaks of IgM has been observed at 28 days of post vaccination as in wolfish and in Atlantic salmon immunized with *A. salmonicida* oil adjuvant vaccine. In this study, immunity was not 100%, and needs to include the immune dominant & the antigens which are not immune protected could be discarded. The use of advanced adjuvants can enhance the efficacy of vaccines (Grontvedt, Lund & Espelid 2004).

Conclusions

Recent developments in biotechnology and the creation of new pathogen vaccines have significantly decreased the danger of disease outbreak and associated losses in aquaculture. The development led to the identification of protective antigens and the safe and affordable production of vaccines. While investigating a cost-effective technology of monitoring serious infection threats, aquaculture vaccination is increasingly becoming an important part of health management. On the other hand, the majority of the research and development work on aquatic animal vaccines is still in its infancy, and issues with multi-component and affordable vaccination programmes have yet to be resolved. The development or commercialization of vaccines for any economically important fish illness is restricted by technical, scientific, biological, and other constraints. We have formulated a vaccine as well as a model of infection for rainbow trout and determine the lethal dose of Ars-Pak-19. The infection caused by this strain was chronic, and initial colonization was observed in the liver and kidney. This vaccine provided comparatively significant protection.

Acknowledgement

The authors are highly thankful to the Department of Fisheries Gilgit Baltistan for execution of this research at their research station Kargah valley. The work presented in this paper is part of a project sponsored by Pakistan Science Foundation (PSF) under grant No. PSF/NSLP/P-UVAS (701).

References

Adams, A. & Subasinghe, R. 2019. Use of fish vaccines in aquaculture (including methods of administration). *Veterinary Vaccines for Livestock*. 1st ed. The Food and Agriculture Organization of the United Nations.

Alonso, M. & Leong, J.A. 2013. Licensed DNA vaccines against infectious hematopoietic necrosis virus (IHNV). *Recent Pat DNA Gene Seq.* 7(1): 62-65.

Arkoosh, M.R., Dietrich, J.P., Rew, M.B., Olson, W., Young, G. & Goetz, F.W. 2018. Exploring the efficacy of vaccine techniques in juvenile sablefish, *Anoplopoma fimbria. Aquac Res*. 49(1): 205-216.

Assefa, A. & Abunna, F. 2018. Maintenance of fish health in aquaculture: Review of epidemiological approaches for prevention and control of infectious disease of fish. *Vet. Med. Int.* 2018: 5432497.

Austin, B. & Austin, D.A. 2016. Aeromonadaceae representative (*Aeromonas salmonicida*). In *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*. Springer, Cham. pp. 215-321.

Austin, B. & Austin, D.A. 2012. Vibrionaceae representatives. In *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*. Springer, Dordrecht. pp. 357-411.

Brown, A.B., Whyte, S.K., Braden, L.M., Groman, D.B., Purcell, S.L. & Fast, M.D. 2020. Vaccination strategy is an important determinant in immunological outcome and survival in Arctic charr (*Salvelinus alpinus*) when challenged with atypical *Aeromonas salmonicida. Aquaculture* 518: 734838.

Brudeseth, B.E., Wiulsrød, R., Fredriksen, B.N., Lindmo, K., Løkling, K.E., Bordevik, M., Steine, N., Klevan, A. & Gravningen, K. 2013. Status and future perspectives of vaccines for industrialised fin-fish farming. *Fish Shellfish Immunol.* 35(6): 1759-1768.

Brummett, R. 2014. *Introduction, Reducing Disease Risk in Aquacultureе*. World Bank Group. pp. 1-9.

Castro, R., Jouneau, L., Pham, H.P., Bouchez, O., Giudicelli, V., Lefranc, M.P., Quillet, E., Benmansour, A., Cazals, F., Six, A. & Fillatreau, S. 2013. Teleost fish mount complex clonal IgM and IgT responses in spleen upon systemic viral infection. *PloS Patho*. 9(1): e1003098.

Chakraborty, S., Cao, T., Hossain, A., Gnanagobal, H., Vasquez, I., Boyce, D. & Santander, J. 2019. Vibrogen-2 vaccine trial in lumpfish (*Cyclopterus lumpus*) against *Vibrio anguillarum*. *Journal of Fish Diseases* 42(7): 1057-1064.

Chandler, D.E. & Roberson, R.W. 2009. *Bioimaging: 2009 Current Concepts in Light and Electron Microscopy.* Massachusetts: Jones & Bartlett Publishers.

Cipriano, R.C. & Bullock, G.L. 2001. *Furunculosis and Other Diseases Caused by Aeromonas salmonicida.* National Fish Health Research Laboratory.

Croisetiere, S., Tarte, P.D., Bernatchez, L. & Belhumeur, P. 2008. Identification of MHC class IIβ resistance/susceptibility alleles to *Aeromonas salmonicida* in brook charr (*Salvelinus fontinalis). Mol. Immunol*. 45(11): 3107-3116.

Dallaire-Dufresne, S., Tanaka, K.H., Trudel, M.V., Lafaille, A. & Charette, S.J. 2014. Virulence, genomic features, and plasticity of *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis. *Vet Microbiol.* 169(1-2): 1-7.

Eslamloo, K., Kumar, S., Caballero-Solares, A., Gnanagobal, H., Santander, J. & Rise, M.L. 2020. Profiling the transcriptome response of Atlantic salmon head kidney to formalin-killed *Renibacterium salmoninarum. Fish Shellfish Immunol*. 98: 937-949.

Fast, M.D., Tse, B., Boyd, J.M. & Johnson, S.C. 2009. Mutations in the *Aeromonas salmonicida* subsp. *salmonicida* type III secretion system affect Atlantic salmon leucocyte activation and downstream immune responses. *Fish Shellfish Immunol.* 27(6): 721-728.

Grontvedt, R.N., Lund, V. & Espelid, S. 2004. Atypical furunculosis in spotted wolffish (*Anarhichas minor* O.) juveniles: Bath vaccination and challenge. *Aquaculture*. 232(1-4): 69-80.

Gudding, R. & Goodrich, T. 2014. The history of fish vaccination. *Fish Vacc.* 12: 1-2.

Gudding, R. & Van Muiswinkel, W.B. 2013. A history of fish vaccination: Science-based disease prevention in aquaculture. *Fish Shellfish Immunol*. 35(6): 1683-1688.

Hnasko, R. 2015. *ELISA, Methods and Protocols*. 1st ed. New York. Humana Press. X: 216.

Holten-Andersen, L., Dalsgaard, I. & Buchmann, K. 2012. Baltic salmon, *Salmo salar*, from Swedish River Lule Älv is more resistant to furunculosis compared to rainbow trout. *PLoS ONE* 7(1): e29571.

Hordvik, I. 2015. Immunoglobulin isotypes in Atlantic salmon, *Salmo salar. Biomolecules* 5(1): 166-177.

Janda, J.M. & Abbott, S.L. 2010. The genus Aeromonas: Taxonomy, pathogenicity, and infection. *Clin. Microbiol. Rev.* 23(1): 35-73.

Kamil, A., Falk, K., Sharma, A., Raae, A., Berven, F., Koppang, E.O. & Hordvik, I. 2011. A monoclonal antibody distinguishes between two IgM heavy chain isotypes in Atlantic salmon and brown trout: Protein characterization, 3D modeling and epitope mapping. *Mol. Immunol.* 48(15-16): 1859-1867.

Kjoglum, S., Larsen, S., Bakke, H.G. & Grimholt, U. 2008. The effect of specific MHC class I and class II combinations on resistance to furunculosis in Atlantic salmon (*Salmo salar*). *Scand. J. Immunol.* 67: 160-168.

Klesius, P.H. & Pridgeon, J.W. 2014. Vaccination against enteric septicemia of catfish. *Fish Vac.* 12: 211-225.

Krkosek, M. 2017. Population biology of infectious diseases shared by wild and farmed fish. *Can. J. of Fish Aquat. Sci.* 74(4): 620-628.

Leboffe, M.J. & Pierce, B.E. 2015. *Microbiology: Laboratory Theory and Application.* Colorado: Morton Publishing Company.

Lee, K.K. & Ellis, A.E. 1991. The role of the lethal extracellular cytolysin of *Aeromonas salmonicida* in the pathology of furunculosis. *J. Fish Dis.* 14(4): 453-460.

Ling, X.D., Dong, W.T., Zhang, Y., Hu, J.J., Liu, J.X. & Zhao, X.X. 2019. A recombinant adenovirus targeting typical *Aeromonas salmonicida* induces an antibody-mediated adaptive immune response after immunization of rainbow trout. *Microbial Patho.* 133: 103559.

Long, M., Zhao, J., Li, T., Tafalla, C., Zhang, Q., Wang, X., Gong, X., Shen, Z. & Li, A. 2015. Transcriptomic and proteomic analyses of splenic immune mechanisms of rainbow trout (*Oncorhynchus mykiss*) infected by *Aeromonas salmonicida* subsp. *salmonicida*. *J. Proteomics.* 122: 41-54.

Lulijwa, R., Alfaro, A.C., Merien, F., Burdass, M., Venter, L. & Young, T. 2019. *In vitro* immune response of chinook salmon (*Oncorhynchus tshawytscha*) peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. *Fish Shellfish Immunol.* 94: 190-198.

Magnadottir, B. 2010. Immunological control of fish diseases. *Marine Biotechnology* 12: 361-379.

Magnadottir, B., Bambir, S.H., Gudmundsdóttir, B.K., Pilström, L. & Helgason, S. 2002. Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. *J. Fish Dis.* 25(10): 583-597.

Mahoney, R.T., Krattiger, A., Clemens, J.D. & Curtiss III, R. 2007. The introduction of new vaccines into developing countries: IV: Global access strategies. *Vaccine* 25(20): 4003-4011.

Mashoof, S. & Criscitiello, M.F. 2016. Fish immunoglobulins. *Biology (Basel)* 5(4): 45.

Midtlyng, P.J. 2016. Methods for measuring efficacy, safety and potency of fish vaccines. In *Fish Vaccines*, edited by Adams, A. *Birkhauser Advances in Infectious Diseases.* Springer, Basel. pp. 119-141.

Mikkelsen, H., Lund, V., Larsen, R. & Seppola, M. 2011. Vibriosis vaccines based on various sero-subgroups of *Vibrio anguillarum* O2 induce specific protection in Atlantic cod (*Gadus morhua* L.) juveniles. *Fish Shellfish Immunol.* 30(1): 330-339.

Parra, D., Takizawa, F. & Sunyer, J.O. 2013. Evolution of B cell immunity. *Annu. Rev. Anim. Biosci.* 1(1): 65-97.

Patterson, H., Saralahti, A., Parikka, M., Dramsi, S., Trieu-Cuot, P., Poyart, C. & Rounioja, S. & Rämet, M. 2012. Adult zebrafish model of bacterial meningitis in *Streptococcus agalactiae* infection. *Dev. Comp. Immunol.* 38(3): 447-455.

PHARMAQ. Alpha Ject micro 4. 2020. https://www.drugs.com/vet/alpha-ject-micro-4- 562 can.html

Pressley, M.E., Phelan III, P.E., Witten, P.E., Mellon, M.T. & Kim, C.H. 2005. Pathogenesis and inflammatory response to *Edwardsiella tarda* infection in the zebrafish. *Dev Comp Immunol.* 29(6): 501-513.

Ramakrishnan, M.A. 2016. Review of the method of "right and wrong cases" ('constant stimuli') without Gauss's formula. *World J. Virol*. 5(2): 85-86.

Ronneseth, A., Ghebretnsae, D.B., Wergeland, H.I. & Haugland, G.T. 2015. Functional characterization of IgM+ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus* L.). *Dev. Comp. Immunol.* 52(2): 132-143.

Sambrook, J. & Russell, D.W. 2001. *Molecular Cloning: A Laboratory Manual.* 3rd ed. New York: Cold Spring Harbor Lab. Press. p. 413.

Santander, J., Golden, G., Wanda, S.Y. & Curtiss III, R. 2012. Fur-regulated iron uptake system of *Edwardsiella ictaluri* and its influence on pathogenesis and immunogenicity in the catfish host. *Infec Immun*. 80(8): 2689-2703.

Santander, J., Mitra, A. & Curtiss III, R. 2011. Phenotype, virulence and immunogenicity of *Edwardsiella ictaluri* cyclic adenosine 3′, 5′-monophosphate receptor protein (Crp) mutants in catfish host. *Fish Shellfish Immunol.* 31(6): 1142-1153.

Shefat, S.H. 2018. Vaccines for infectious bacterial and viral diseases of fish. *J. Bacteriol. Infec.* 2(2): 1-5.

Shoemaker, C.A., Klesius, P.H., Evans, J.J. & Arias, C.R. 2009. Use of modified live vaccines in aquaculture. *J. World Aqua Soc.* 40(5): 573-585.

Skugor, S., Jørgensen, S.M., Gjerde, B. & Krasnov, A. 2009. Hepatic gene expression profiling reveals protective responses in Atlantic salmon vaccinated against furunculosis. *BMC Genomics* 10(1): 1-5.

Sommerset, I., Krossøy, B., Biering, E. & Frost, P. 2005. Vaccines for fish in aquaculture. *Exp. Rev. Vac.* 4(1): 89-101.

Starliper, C.E. 2011. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum. J. Adv. Res.* 2(2): 97-108.

Sughra, F., Rahman, M., Abbas, F. & Altaf, I. 2021. Evaluation of three alum-precipitated *Aeromonas hydrophila* vaccines administered to *Labeo rohita, Cirrhinus mrigala* and *Ctenopharyngodon idella*: Immunokinetics, immersion challenge and histopathology. *Brazilian Journal of Biology* 83: e249913.

Sundvold, H., Ruyter, B., Østbye, T.K. & Moen, T. 2010. Identification of a novel allele of peroxisome proliferator-activated receptor gamma (PPARG) and its association with resistance to *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar). Fish Shellfish Immunol*. 28(2): 394-400.

Valderrama, K., Soto-Dávila, M., Segovia, C., Vásquez, I., Dang, M. & Santander, J. 2019. *Aeromonas salmonicida* infects Atlantic salmon (*Salmo salar*) erythrocytes. *J. Fish Dis.* 42(11): 1601-1608.

Watts, J.E., Schreier, H.J., Lanska, L. & Hale, M.S. 2017. The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions. *Mar Drugs.* 15(6): 158.

Yang, B., Duan, H., Cao, W., Guo, Y., Liu, Y., Sun, L., Zhang, J., Sun, Y. & Ma, Y. 2019. Xp11 translocation renal cell carcinoma and clear cell renal cell carcinoma with TFE3 strong positive immunostaining: Morphology, immunohistochemistry, and FISH analysis. *Modern Pathology* 32(10): 1521-1535.

Yin, X., Mu, L., Fu, S., Wu, L., Han, K., Wu, H., Bian, X., Wei, X., Guo, Z., Wang, A. & Ye, J. 2019. Expression and characterization of Nile tilapia (*Oreochromis niloticus*) secretory and membrane-bound IgM in response to bacterial infection. *Aquaculture* 508: 214-222.

Zhang, Z., Niu, C., Storset, A., Bøgwald, J. & Dalmo, R.A. 2011. Comparison of *Aeromonas salmonicida* resistant and susceptible salmon families: A high immune response is beneficial for the survival against *Aeromonas salmonicida* challenge. *Fish Shellfish Immunol.* 31(1): 1-9.

*Corresponding author; email: mhafeezurehman@uvas.edu.pk