Sub-Project ID: 030

Program Based Research Grant (PBRG)

Sub-project Completion Report
on
Investigation and Characterization of Viral and Bacterial Diseases in Selected Fin Fishes and Shrimp in Bangladesh and Development of their Vaccines and Validation

Sub-project Duration
27 December 2017 to 20 December 2021

Coordinating Organization

Fisheries Division
Bangladesh Agricultural Research Council
Farmgate, Dhaka 1215

Project Implementation Unit
National Agricultural Technology Program Phase II Project
Bangladesh Agricultural Research Council
Farmgate, Dhaka 1215

October 2021

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Implementing Organization

Fish Health Management Division
Freshwater Station
Bangladesh Fisheries Research Institute, Mymensingh 2202

Department of Microbiology and Hygiene
Faculty of Veterinary Science
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Project Implementation Unit
National Agricultural Technology Program-Phase II Project
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Acknowledgement

The execution of PBRG sub-project has successfully been completed by Fish Health Management Division, Freshwater Station, BFRI, Mymensingh (*Comp 1*) and Department of Microbiology and Hygiene Faculty of Veterinary Science, Bangladesh Agricultural University(BAU) Mymensingh-2202 (*Comp 2*) using the research fund of WB, IFAD and GoB through Ministry of Agriculture. We would like to acknowledge to the World Bank for arranging the research fund and supervising the PBRGs by BARC. It is worthwhile to mention the cooperating and quick responses of PIU-BARC, NATP-2 in respect of field implementation of the sub-project in multiple sites. Preparing the sub-project completion report required to contact a number of persons for collection of information and processing of research data. Without the help of those persons, the preparation of this document could not be made possible. All of them, who have made it possible, deserve apperceptions. Our thanks are due to the Director, PIU-BARC, NATP-2 and his team who given their wholehearted support to prepare this document. We hope this publication would be helpful to the agricultural scientists of the country for designing their future research projects in order to technology generation as well as increasing production and productivity for sustainable food and nutrition security in Bangladesh. It would also assist the policy makers of the agricultural sub-sectors for setting their future research direction.

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Abbreviation and Acronyms

%	Percentage	MR	Methyl-Red
˚C	Celsius	NA	Nutrient Agar
µl	Micro litter	NaCl	Sodium Chloride
ADH	Arginine Di hydrolase	NATP	National Agricultural Technology Project
AHPND	Acute Hepatopancreatic Necrosis Disease	nm	Nano meter
ANOVA	Analysis of variance	NO_2	Nitrogen Dioxide
BAB	Blood Agar Base	NO_3	Nitrate
BARC	Bangladesh Agricultural Research Council	O/F	Oxidative-Fermentative
BAU	Bangladesh Agricultural University	O/W	oil-in-Water
BCSIR	Bangladesh Council of Scientific and Industrial Research	OD	Optical Density
BFRI	Bangladesh Fisheries Research Institute	OF	Oxidative-Fermentative
BHIA	Brain-Heart Infusion Agar	OIE	Office International des Epizooties
BHIB	Brain Heart Infusion Broth	PBRG	Program Based Research Grant
Bp	Base pair	PBS	Phosphate-Buffered Saline
bwt	body weight	PBST	Phosphate Buffer Saline Tween-20
CAMP	Christie–Atkins–Munch-Peterson test	PCR	Polymerase chain reaction
CFU	Colony-forming unit	PhD	Doctor of Philosophy
Co-PI	Co-Principal Investigator	PI	Principal Investigator
cRBC	chicken Red Blood Cell	PIM	Project Implementation Manual
CRG	Competitive Research Grant	PIU	Project Implementation Unit
DEPC	Diethylpyrocarbonate	PP	Protection Profile
DLS	Department of Livestock Services	PUFA	Polyunsaturated Fatty Acids
DMRT	Duncan's Multiple Range Test	RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid	rpm	Revolutions Per Minute
DO	Dissolved oxygen	rpoB	DNA-Directed RNA Polymerase Subunit Beta
DoF	Department of Fisheries	RPS	Relative Percentage Survival
EDTA	Ethylenediamine Tetra acetic Acid	rRNA	Ribosomal RNA
ELISA	Enzyme-linked Immunosorbent Assay	RT-PCR	Reverse Transcription Polymerase Chain Reaction
etc	*Et cetera*	S/C	Sub-Cutaneous
EUS	Epizootic Ulcerative Syndrome	SHT	Syncytial Hepatitis in Tilapia
FAO	Food and Agriculture Organization	SoE	Statement of Expenditure
FY	Financial Year	SOP	Standard Operating Procedure
g	Gram	SPSS	Statistical Package for the Social Sciences
GBS	Group B Streptococcus	SSO	Senior Scientific Officer
GDP	Gross Domestic Product	TAC	Total Aeromonas Count

GILT	Interferon-γ-Inducible Lysosomal Thiol Reductase	TAE	Tris-acetate-EDTA
GoB	Government of Bangladesh	TAN	Total Ammonia Nitrogen
HOPE	Head of Procuring Entity	TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose Agar
IFAD	International Fund for Agricultural Development	TiLV	Tilapia Lake Virus
IgA	Immunoglobulin A	TSA	Tryptic Soy Agar
IgY	Immunoglobulin Y	TSB	Tryptic Soy Broth
IM	Intramuscular	TVC	Total Viable Count
IP	Intraperitoneal	UIA	Unionized Ammonia
IT	Income Tax	UIA-N	Un-ionized Ammonia Nitrogen
kb	kilobase	USA	United States of America
LoA	Letter of Agreement	USB	Universal Serial Bus
M&E	Monitoring and Evaluation	UV	Ultra-Violate
MD	Member Director	V. koi	Vietnamese Koi
mg	Milligram	VAT	Value Added Tax
MH	Mueller Hinton	VP	Voges-Proskauer
ml	Milliliter	WOA	World Organization for Animal Health
mmol/L	Millimoles per liter	W/O/W	Water-in-Oil-in-Water
MPN	Maximum Probable Number	WB	World Bank

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Executive Summary

Aquaculture in Bangladesh is growing rapidly with respect to both quantity and variety of culture fish species. From 2000 and 2016, aquaculture production increased from 712,640 to 2,060,408 metric tons which is much larger quantity than capture production (1.023 million tons) in 2015. The current trend in aquaculture development is towards increased intensification and commercialization of aquatic products. However, Tilapia (*Oreochromis niloticus*), Vietnami Koi (*Anabas testudineus*), Shing (*Heteropneustes fossilis*), Magur (*Clarias batrachus*) Pangas (*Pangasius hypophthalmus*) and Shrimp (*Penaeus monodon*) are being cultured in ponds and ghers mostly as commercial basis by entrepreneurial farmers in Bangladesh. But recently, most of the farmers reported that they are not getting enough profit from freshwater fin fishes and Shrimp culture because of disease occurrence and mortality. Exact causative of such mortality of cultured fishes during the year 2014-2017 were unknown. Similar report of mortality of cultured fin fishes and Shrimp were reported by many other countries of Asia. In most cases they found that the deadly bacteria and viruses are responsible for huge mortality of fin fishes and Shrimp in pond/lake/gher cultured system. The affected countries were able to identify the exact causal agents from the dead and moribund fishes and controlled cultured fish mortality by the development and uses of effective vaccines against those pathogenic bacteria and viruses. As many hatchery owners of Bangladesh are importing brood fin fishes from several countries of Asia, so there is a chance of random introduction of many pathogenic bacteria and viruses with the imported broods or the multi-drug resistant pathogenic bacteria and viruses those might have been evolved from the cultured environment and emerged as deadly diseases of such high valued cultured fin fishes. By adapting the advance technology for the isolation, identification and development of effective vaccines for fishes against the circulating bacteria and viruses many of the deadly infection diseases of cultured fin fishes and Shrimp could easily be controlled in Bangladesh too.

Under the circumstances stated, the present research has been taken to know the fin fish and Shrimp culture strategies, influences of the environmental and host factors and to investigate their diseases occurrence pattern and their health management problems in pond aquaculture through field observation and to isolate and characterize existing and emerging viral and bacterial agents from infected different species of fin fish and shrimp and the research also aimed to also develop and validation of highly effective fish vaccines with the locally circulating bacterial and viral isolates from the newly emerged infectious diseases with a view to establish better health management and increase productivity practices of highly consumed fin fishes and shrimp for Bangladesh.

Under the study, a total of six different species of finfishes namely, Tilapia, Koi, Shing, Magur, Pangas and Shrimp (Bagda) those are considered as highly consumed fishes are become affected with deadly bacterial and viral agents and causing huge morbidity and mortality were selected. Deadly bacterial disease of Tilapia, Koi, Shing, Magur and Pangas fishes known as Epizootic Ulcerative Syndrome (EUS) and Popped eye disease outbreak had been reported from cultured ponds of four different districts namely Mymensingh, Gazipur, Netrokona and Kishoreganj during January to December 2018. Due to Epizootic Ulcerative Syndrome (EUS) of Shing, Magur, Koi, Tilapia and Pangas; mass mortality of the fish had been reported from each of the said districts during the outbreak period.

Freshly dead and moribund Shing, Magur, Koi, Tilapia and Pangas fishes were collected from affected ponds of different districts. Four different types of highly pathogenic bacteria species (*Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus*) have been isolated from the above mentioned six different species of fishes.

The isolated bacterial species then characterized for the fishes suffering from exophthalmia (Pop-eye), EUS or Red Spot Disease (RSD) and Early Mortality Syndrome (EMS)/ Acute Hepatopancreatic Necrosis Disease (AHPND) using various cultural, morphological, biochemical and molecular tests. The isolated bacteria revealed identical sign, symptoms and deathpatternintheexperimentallyinducedinfectedhealthyShing,Magur, Koi, Tilapia and Pangas fishes in aquarium-based infection. The death pattern and symptoms oftheartificiallyinfectedfishesweresimilartothatofthediseasesymptomsoffieldoutbreak. Later on an inactivated whole cell bacterial vaccines (mono-, bi- and tri-valent) were developed using the field isolates of bacteria with aluminum hydroxide gel with mineral oil and the vaccines were injected through I/M, IP and oral routes into the healthy adult Shing, Magur, Koi, TilapiaandPangasfishesatadoseof0.1,0.3and0.5ml/fishintheaquariumatFish Disease and Health Management laboratory, BFRI for each vaccine. Fishes immunized with 0.3 and 0.5 ml/fish with the newly developed vaccines of each type were able to protect 100% fish (each type of fish) mortalityfromexperimentallyinducedchallengeinfectionwithhomologousbacterialisolates.

Each species of the immunized fishes was subjected to challenge experiment at the BRFI Fish Disease and Health Management laboratory (aquarium based) after completion of two doses of vaccination with each type of vaccine and the fishes were challenged with the homologus bacterial strains (*S. agalactiae, A. hydrophilus, A. veronii* and *V. parahaemolyticus*) at a dose of 6.3 x10^6CFU/ml, 1.3 x10^6CFU/ml, 2.3 x10^6 CFU/ml and 8.6 x10^6CFU/ml respectively.

Meanwhile immunization of four laying hens (total of sixteen along with four control hens) for each type of fish vaccine has also been inoculated simultaneously at the laboratory of small animal house, Dept. of Microbiology and Hygiene, BAU, Mymensingh.

Primary vaccination was done in the four different species (Shing, Magur, Koi and Tilapia) of cultured brood female and male fishes of two different hatcheries of which three species of fishes were (Shing, Magur and Koi) at Ma Motsho Hatchery at Dohakhola, Gouripur, Mymensingh, one species of fish (Tilapia) at the M.O. Agro-Fisheries and Hatchery, Bagan, Trishal, Mymensingh and adult pangas fish of BFRI fresh water station, Mymensingh all the fishes were vaccinated with 0.3 ml/ fish of each vaccine (mono-valent, bi-valent and tri-valent vaccines) with double doses.

Fry developed from the vaccinated brood of each species of fishes were fed with the egg yolk mixed with flour and skimmed milk of the immunized hen for a period of 7 days, three consecutive days starting from day 4^{th} today 6^{th} of fry's age in the cistern and at day 7^{th} today 10^{th} in the nursery ponds with a view to transfer the immunoglobulins (IgM and IgY) raised against each bacterial antigens from the eggs of immunized hens those were vaccinated with newly developed fish vaccines. After successful completion of immunization of fishes at the hatchery/ ponds level by following passive immunization with feed-based fish vaccines, a challenge experiment was conducted using representative number of fishes (ten fishes of each species for each type of vaccine used for immunization) in the laboratory of BFRI aquarium. Isolation and characterization of Tilapia Lake Virus (TiLV) were successfully completed at the laboratory level but preparation of vaccine and field trials were yet to be done.

From the date of commencement of the sub-project, four mono-valent, one bi-valent and two tri-valent inactivated bacterial vaccines for Shing, Magur, Koi, Tilapia and Pangas fishes has been developed and their validation at laboratory and field level were successfully completed to achieve the goal of the present research.

Keywords: Epizootic Ulcerative Syndrome, Early Mortality Syndrome, Acute Hepatopancreatic Necrosis Disease, Immunoglobulin.

PBRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the PBRG sub-project

Investigation and Characterization of Viral and Bacterial Diseases in Selected Fin fishes and Shrimp in Bangladesh and Development of their Vaccines and Validation

2. Implementing organization (s)

Fish Health Management Division
Freshwater Station, Bangladesh Fisheries Research Institute
Mymensingh-2202 (Comp 1), and

Department of Microbiology & Hygiene
Faculty of Veterinary Science
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4. Sub-project budget (TK)

4.1. Total budget (in Tk. as approved)**:** Tk. 24798004.00 *(Taka Two crore forty seven lac ninety eight thousand four)*

BARC component: Tk. 24,20000.00
BFRI component: Tk. 76,54,512.00
BAU component: Tk. 1,57,23,492.00

4.2. Latest Revised budget (if any)**:** Not applicable

5. Duration of the sub-project: 4 years

5.1. Start date (based on LoA signed)**:** 27 December 2017
5.2. End date**:** 20 December 2021

6. Background of the sub-project

Aquaculture in Bangladesh is growing rapidly with respect to both quantity and variety of fish species. From 2000 and 2016, aquaculture production increased from 712,640 and 2,060,408 metric tons which is much larger quantity than wild capture production (1.023 million tons) in 2015 (Shamsuzzaman *et al.*, 2017). Pond culture represents the mainstay of aquaculture in Bangladesh, accounting for 85.8% of the total recorded production and 57.7% of the area under culture (DoF, 2010). The current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. However, Tilapia (*Oreochromis niloticus*), Koi (*Anabas testudineus*), Shing (*Heteropneustes fossilis*), Magur (*Clarias batrachus*) Pangas (*Pangasius hypophthalmus*) and Shrimp (*Penaeus monodon*) are being cultured in ponds mostly as commercial basis by entrepreneurial farmers in Bangladesh.

Among different freshwater fin fishes, Tilapia (*O. niloticus*), Koi (*A. testudineus*), Shing (*H. fossilis*), Magur (*C. batrachus*) Pangas (*P. hypophthalmus*) and Shrimp (*P. monodon*) and are very popular and high valued fish species in Bangladesh. The air-breathing catfishes, particularly shing is not only recognized for its excellent taste and market value but also sought highly for its nutritional and medicinal values (Rahman *et al.* 2013). Although, Shing, Tilapia, Koi and Magur culture has great potential in Bangladesh but unknown emerging diseases are causing serious economic losses because of their high mortality within 3 to 10 days under farming conditions. There are several viral, bacterial, parasitic and fungal diseases affecting total growth period (fry to adult) and their productivity in pond culture system.

Most of the viral and bacterial diseases which have not been detected or characterized and their control/preventive measures (through vaccine) are not available yet in Bangladesh. Freshwater fin fishes specially Tilapia (*Oreochromis niloticus*), Koi (*Anabas testudineus*), Shing (*Heteropneustes fossilis*), Magur (*Clarias batrachus*) Pangas (*Pangasius hypophthalmus*) and Shrimp (*Penaeus monodon*) fishes all over the world are being affected with the viruses of different families such as Rabdoviridae, Orthomyxoviridae, Alloherpesviridae, Iridoviridae and Nodaviridiae (Bowser *et al.*, 1985).

Among the infectious diseases, Bangladesh has the experiences of several outbreaks of bacterial and viral diseases in cultured Tilapia. Recently mass mortality of Tilapia has brought under the noticed of DoF and BFRI due to unknown causes other than bacteria and virus. As different hatchery owners are importing brood Tilapia from different countries of Asia every year under a process of weak no quarantine measure and thus various imported causative agents entering the country and diseases occurring severely in Tilapia and many other fishes. In most of such cases causes mass mortality within few days showing characteristic clinical signs of gasping, haemorrhage at fins and other part of the body, scale protrusion and circulating movement and post mortem lesions (enlarge and pale liver, inflamed kidney, spleen, gall balder and thin watery blood) in the internal organs of the death and moribund Tilapia. Different outbreaks of diseases without isolation of bacteria and virus indicating the cause of acute death of huge number of Tilapia in a short time might be due to Tilapia Lake Virus (TiLV). However, recently TiLV outbreaks among cultured Tilapia have occurred in Thailand, Israel, Ecuador, Colombia and Egypt wherein high cumulative mortalities (20-90%) were observed and recorded (Dong *et al.*, 2017). This has been reported as an emerging viral disease which is responsible for causing Syncytial Hepatitis in Tilapia (SHT). The etiological agent causing this mass mortality has been described and identified as a novel Orthomyxo-like (RNA) virus named as Tilapia lake virus (TiLV) (Eyngor *et al.* 2014; Bacharach *et al.*, 2016). On the other hand, Streptococcosisinfection is one of the major bacterial disease affecting farmed Tilapia (*O. niloticus*) and Koi (*A. testudineus*) worldwide which is also responsible for high mortality up to 70-90% over a period of 7-10 days and also responsible for high economic losses (Wongsathein, 2012).

Despite multiple approaches to innovative therapy, fish diseases remain a major economic issue in commercial aquaculture worldwide. Although antibiotics or chemotherapeutics may be implemented for disease treatment, there are some clear drawbacks, such as drug resistance issues and safety concerns (Sneeringer *et al*. 2019). Given the development of new technology and a lack of research reviews on advancements in fish vaccine technologies, there is a need for a comprehensive overview of where the field is currently. However, the first reported use of a fish vaccine in Bangladesh was a killed *Streptococcous agalactiae* vaccine namely *Koi Vac* jointly produced by Bangladesh Fisheries Research Institute and Department of Microbiology and Hygiene, BAU for Vietnamese Koi, *Anabas testudineus*. (BFRI Final Report, 2019).

Shrimp (*Penaeus monodon*) particularly Bagda is another leading foreign currency earning product of Bangladesh. Only by exporting Shrimp, Bangladesh is earning around 630.24 million dollar per year and contributing 3.78% in GDP (DoF, 2015). However, Acute Hepatopancreatic Necrosis Disease Syndrome (AHPND), formally known as shrimp early mortality syndrome, has recently caused serious problems in the shrimp culture industry. It occurs most frequently within the first 30 days after stocking a newly prepared shrimp pond. This disease was first reported in

China in 2009 and then from Vietnam, Malaysia and Thailand (Zorriehzahra and Banaederakhshan, 2015). In early 2013, the causal agent of AHPND was identified as a unique isolates of *Vibrio parahaemolyticus* (Tran *et al.*, 2008). AHPND reduces Shrimp production and causes a huge economic loss for the global shrimp industry.

Recently, Shrimp culture industry in Bangladesh has also been facing similar pattern of diseases which is responsible for mass mortally of shrimp showing symptoms of AHPND as mentioned in shrimp diseases of China, Vietnam, Malaysia and Thailand. Although, AHPND has not been confirmed yet in shrimp industry of Bangladesh but then considering as an emerging disease of Shrimp which might be responsible for high mortality and causing serious economic losses to the farmers. The etiological agents of this disease of shrimp need to be identified and an effective preventive measure should be taken against the disease to sustain the productivity of shrimp culture in Bangladesh

Keeping in consideration all the stated issues of the sector, thus the principal objectives of this study has been set to investigate the various environmental factors (water temperature, pH, dissolved oxygen, and quantities of unionized ammonia in the water) influencing the occurrence of infectious diseases, determine the pathogenicity of the infectious agents

- To isolate, identify and characterize the isolated emerging and existing viruses and bacteria responsible for mass mortality of fin fishes and Shrimp
- To Development of effective fish vaccines and to evaluate efficacy of the experimentally developed vaccines in the specific hosts with the isolated viruses and bacteria

7. Sub-project general objective

To control and reduce mass mortality of fin and shell fish due to bacterial and viral diseases and characterization the causative agents through biological and molecular methods to establish better health management practices in fin and shell fish aquaculture with increased productivity.

8. Sub-project specific objectives (Component wise)

Coordination component (BARC)

- To ensure smooth and efficient implementation of sub-project activities to achieve desired sub-project outputs within the stipulated timeframe under strengthened capable research management system;
- To coordinate sub-project implementation efforts and integration of activities to generate desired information /technology as per methodology of the sub-projects;
- Identify operational deviations and addressing constraints/problems (if any) under a process of strong and regular monitoring of the sub-project activities;
- To upgrading the level of output of the sub-project through reviewing of yearly technical progress;
- Collect and collate sub-project data, finding and observation and production of compiled Project Completion Report (PCR).

Component-1 (BFRI)

- To investigate the various environmental factors (water quality, fish density, cultured pattern, age of fish species and influence of the seasons) influencing the occurrence of infectious diseases of cultured fin fish (Shing, *Heteropneustes fossilis*; Tilapia, *Oreochromis niloticus;* Koi, *Anabas testudineus* and Gulsha, *Mystus vittatus*) and Shrimp, *Penaeus monodon* in Bangladesh.
- To determine the pathogenicity of the field isolates of bacteria and viruses by inducing aquarium based experimental infection using different fin fishes;
- To validate the efficacy of experimentally developed fish vaccines against five different species of fin fishes at the laboratory and at farm level.

Component-2 (BAU)

- To isolate and identify emerging and existing viruses and bacteria responsible for mass mortality of fin fishes and shrimp in Bangladesh;
- To detect and characterize the isolated viruses and bacteria and also to know their origin by using biological and molecular methods (RT-PCR/PCR/nt/aa sequencing and construction of phylogenetic tree of specific genes of each isolate);
- To develop an effective fish vaccines with the isolated viruses and bacteria against six different species of fin fishes and shrimp prevalent in Bangladesh;
- To evaluate efficacy of the experimentally developed fish vaccines in the specific hosts (five kinds of fin fishes) through laboratory and field trials.

9. Implementing location(s)

The sub-project implementation location is at Freshwater Station, BFRIBFRI, Mymensingh (*Comp-1*) and at the Dept. of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202 (*Comp-2*). Field research activities of the sub project included Trishal and Gouripur experimental fish farms areas of Mymensingh districts.

10. Methodology in brief

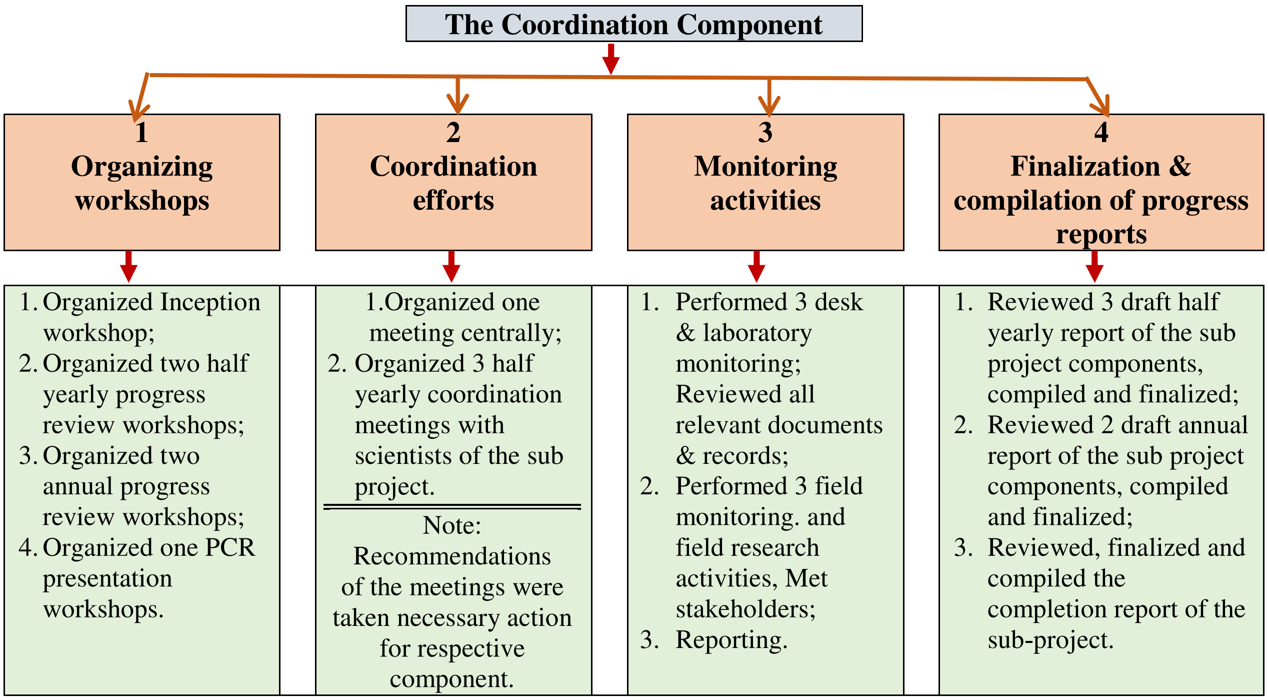
10.1. Activity implementation approach of the Coordination component

The Coordination component as the responsible unit of the sub project to initiate all potential efforts in the process of implementation of each component under the sub project so that the general objectives and goal of the sub-project can be achieved through smooth and successful completion of each of the specific objectives as per activity time plan of thesub- project. To ensure that, the Coordination component, taken into consideration its own activity and objectives and duration of the sub-project, thus accordingly designed its own plan of activity (approach) for the proposed period.

Following are the major activities carried out by the Coordination component under the plan:

a. Organizing seminars/workshops.
b. Monitoring the sub-project activities (specifically financial and research activities);
c. Coordination activities within the component sub-projects.
d. Review and compilation of half yearly and annual research progress reports;

The implementation approach and activities thereunder for the Coordination component of the sub-project shown in the following diagram:



Recommendations of the inception, half yearly and annual research progress review workshops and different coordination meetings are furnished hereunder in **Appendices- BARC: A - D.**

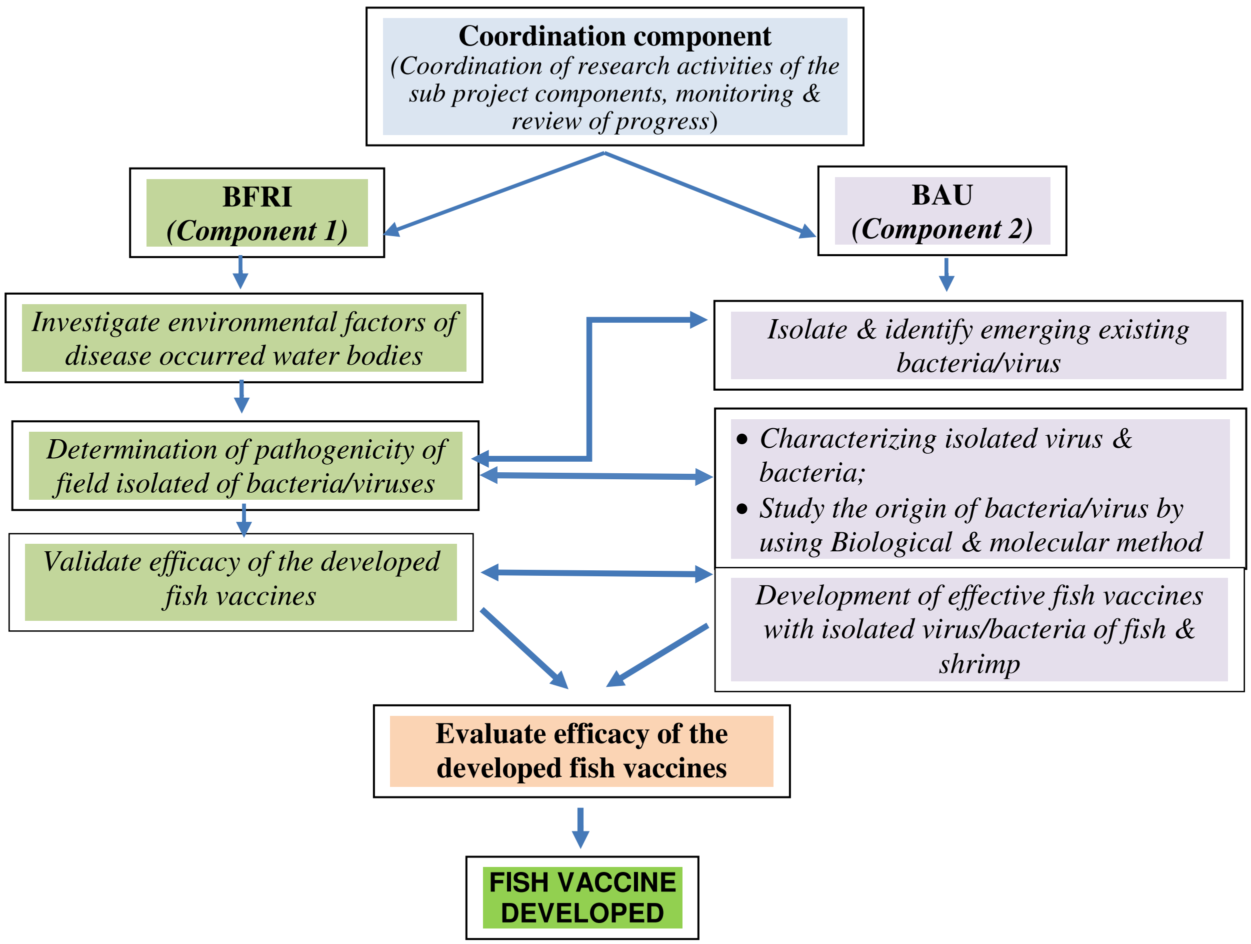
Following table presenting the summary statement of achievements performed by the Coordination component of the sub project:

Summary statement of achievements		
Name of activities	**Performance against each activity**	**Remark**
Inception workshop	Organized centrally at BARC in November' 2018	Attended all PI, Co-PI & expert members.
Revision of PP	Done as per recommendations of Inception Workshop	-
Half yearly progress review workshop (Date)	Organized centrally at BARC in March' 2019 and January'2020.	Attended all PI, Co-PI & expert members
Annual progress review workshop (Date)	Organized centrally at BARC in July' 2019 & in September' 2020	Attended all PI, Co-PI & expert members.
Coordination meeting (No)	03 07.02.19, 19.10.19 & 25.06.20	One Coordination meeting held centrally.
Monitoring of field and Lab activities	04 (BFRI & BAU)	Covered all components under sub-project.
Financial achievement	Approx. 47%of released money & 19%of total approved budget	-
Reporting performance	Provided sub-project inception report, SoE, Half yearly and Annual compiled progress reports of all sub project components as per planned time frame.	Major reports are: • Inception report (1 no) • Compiled half yearly progress report (2 no) • Compiled annual progress report (2 no). • Monitoring reports (3 no).

Pictorial views of different workshops, coordination meetings and field monitoring activities

10.2. Research approach

The two research components (namely, BFRI & BAU) of the sub-project followed the prescribed approach of works implemented under the strong coordination and monitoring efforts of the BARC Fisheries Division as Coordination Unit so that a fruitful output can be achieved within the stipulated timeframe of the research sub-project. The approach of work and implementation procedure and research linkage of the components are presented in the following flow diagram.



Flow diagram 1. Coordination approach of the sub project research components toward development of fish vaccines.

10.3. Baseline study on health and diseases of cultured fin fishes and shrimp

Infectious diseases in aquaculture are of major concern to the industry and are typically controlled by eradication of the pathogen, treatment with antibiotic or chemotherapeutics, and/or by preventative measures such as the use of probiotics or vaccines. The purpose of the baseline study was to know the prevalence of pathogenic bacterial and viral diseases of fin fishes and shrimp responsible for mass mortality in some selected districts of Bangladesh. The baseline survey was conducted for Tilapia, V. Koi, Shing, Magur and Pangas fishes in Mymensingh, Netrokona, Gazipur, Narsingdi, Kishoreganj and Chandpur districtsin the year 2018 by using a pretested questionnaire. Survey areas for baseline data collection was initially done in consultation with the respective departmental officials of the greater Mymensingh district and Chandpur District. Then screening of the areas was done on the basis of degree of fish disease occurrence during the previous consecutive four years. Secondary data recording on disease occurrence and mortality of fish in those were also recorded covering the period from 2014-

2017. In case of shrimp, the survey was conducted in selected costal ghers of Bagerhat, Satkhira and Khulna districts.

10.4. Water quality determination

Fish and other organisms with cultural potential live in water, thus, it is no surprise that professional fish culturists state that "Water quality determines to a great extent the success or failure of a fish cultural operation" (Piper *et al.,* 1982). In this study we considered mainly water temperature, Dissolved Oxygen, pH and unionized ammonia. Boyd (1990 a and b) are excellent references on water quality and water quality management for aquaculture. For this experiment HACH Kit FF3 was used for measuring the mentioned water quality parameters.

Plate 1. Water quality analysis at field

10.4.1. Water temperature

Temperature has a large effect on the chemical and biological systems in water. Lower temperatures decrease the growth rates of fish and fish food organisms but increase the solubility of oxygen. Higher temperatures increase the use of dissolved oxygen by aquatic life but decrease the solubility of oxygen. Different temperature zones occur in natural waters because the density of water changes with temperature. Fish can adjust to different temperature zones if the temperature changes slowly. Sudden changes in water temperature can kill fish. It was measured by Max-min thermometer

10.4.2. Water pH

The pH of water is a measure of the hydrogen ion concentration on a scale of 0 (very acidic) to 14 (very alkaline), with pH 7 being neutral. The pH of most natural waters ranges from pH 4 to pH 9. Dissolved Carbon dioxide, carbonates, bicarbonates and acid rain cause changes in the pH. Phytoplankton and other aquatic plant life remove Carbon dioxide from the water during photosynthesis, which causes the pH to increase during the day. To make an estimate of the pH cycle of a body of water, make pH measurements at different times of the day. The best pH values for fish production measured at sunrise are approximately 6.5 to 9. The acid and alkaline death points for most fish are approximately pH 4 and pH 11. Wide range pH indicator solution with colour disc, box and plastic viewing tubes of HACH kit FF3 was used in this study.

10.4.3. Dissolved Oxygen

Oxygen is the first limiting factor for growth and well-being of fish. Fish require oxygen for respiration, which physiologists express as mg of oxygen consumed per kilogram of fish per hour ($mgO_2/kg/h$). The respiratory rate increases with increasing temperature, activity, and following feeding, but decreases with increasing mean weight. To measure the dissolved oxygen, the sample from a tank or pond, hold the bottle with the stopper in the water, then remove the stopper and let the bottle fill. Remove and drain the bottle, add the stopper and fill the bottle again. The chemical used for this were Dissolved Oxygen 1, 2, 3 which were available in the Hach kit FF3 box.

10.4.4. Unionized Ammonia in water

The major source of ammonia in a water of a heavily stocked culture pond is from excretion of fish, mostly via their gills. Ammonia is produced by animals as a by-product of protein metabolism. It is generally measured by chemical analysis (Nessler method) which is called total ammonia nitrogen (TAN) because it includes two forms of ammonia:ammonia (NH_3), the unionized form, and the ammonium ion (NH_4^+). The unionized ammonia (UIA) is toxic to fish. To calculate the mg/L NH_3 in the sample, refer to chart and the equation according to Thurston *et al.,* (1977). The calculated equation is as follows:

$$\text{mg/L } NH_3 = ((\text{mg/L } NH_3\text{–N x percent } NH_3 \text{ from chart}) \div 100) \times 1.2$$

Chart for calculating NH_3 (mg/L) following equation according to Thurston et al. (1977)

pH	16ºC	18 ºC	20 ºC	22 ºC	24 ºC	26 ºC	28 ºC	30 ºC	32 ºC
7	0.29	0.34	0.39	0.46	0.52	0.6	0.69	0.8	0.91
7.2	0.46	0.54	0.62	0.82	0.83	0.96	1.1	1.26	1.44
7.4	0.73	0.85	0.98	1.14	1.31	1.5	1.73	1.98	2.26
7.6	1.16	1.34	1.55	1.79	2.06	2.36	2.71	3.1	3.53
7.8	1.82	2.11	2.44	2.81	3.22	3.7	4.23	4.82	5.48
8	2.86	3.3	3.81	4.38	5.02	5.74	6.54	7.43	8.42
8.2	4.45	5.14	5.9	6.76	7.72	8.8	9.98	11.29	12.72
8.4	6.88	7.9	9.04	10.31	11.71	13.26	14.95	16.78	18.77
8.6	10.48	11.97	13.61	15.41	17.37	19.5	21.78	24.22	26.8
8.8	15.66	17.73	19.98	22.41	25	27.74	30.62	33.62	36.72
9	22.73	25.46	28.36	31.4	34.56	37.83	41.16	44.53	47.91
9.2	31.8	35.12	38.55	42.04	45.57	49.09	52.58	55.99	59.31
9.4	42.49	46.18	49.85	53.48	57.02	60.45	63.73	66.85	69.79
9.6	53.94	57.62	61.17	64.56	67.77	70.78	73.58	76.17	78.55
9.8	64.99	68.31	71.4	74.28	76.92	79.99	81.53	83.51	85.3
10	74.63	77.35	79.83	82.08	84.04	85.88	47.49	88.92	90.19
10.2	82.34	84.41	86.25	89.33	89.33	90.6	91.73	92.71	93.55

10.5. Selection of sampling areas for the isolation and identification of pathogenic bacteria from fin fishes and shrimp

Samples were collected from outbreak site of the selected areas of Mymensingh, Gazipur, Netrokona, Kishoreganj, Bagerhat, Khulna and Satkhira districtsof Bangladesh. A total number of 40 ghers/ponds from 15 disease out breaked upazilas of the seven districts were covered (Fig. 1).

Name of Districts	Name of Upazila	Pond / Gher	Total Pond/ Gher
Mymensingh	Trishal	8	15
	Gouripur	3	
	Tarakanda	2	
	Phulpur	1	
	Muktagacha	1	
Netrokona	Purbadhala	5	08
	Kendua	3	
Kishoreganj	Hossainpur	3	05
	Pakundia	2	
Gazipur	Sreepur	3	07
	Kaliakair	3	
	Kapasia	1	
Bagerhat	Bagerhat sadar	3	05
Khulna	Paikgachha	1	
Satkhira	Satkhira Sadar	1	
7 Districts	15 Upazilas		40

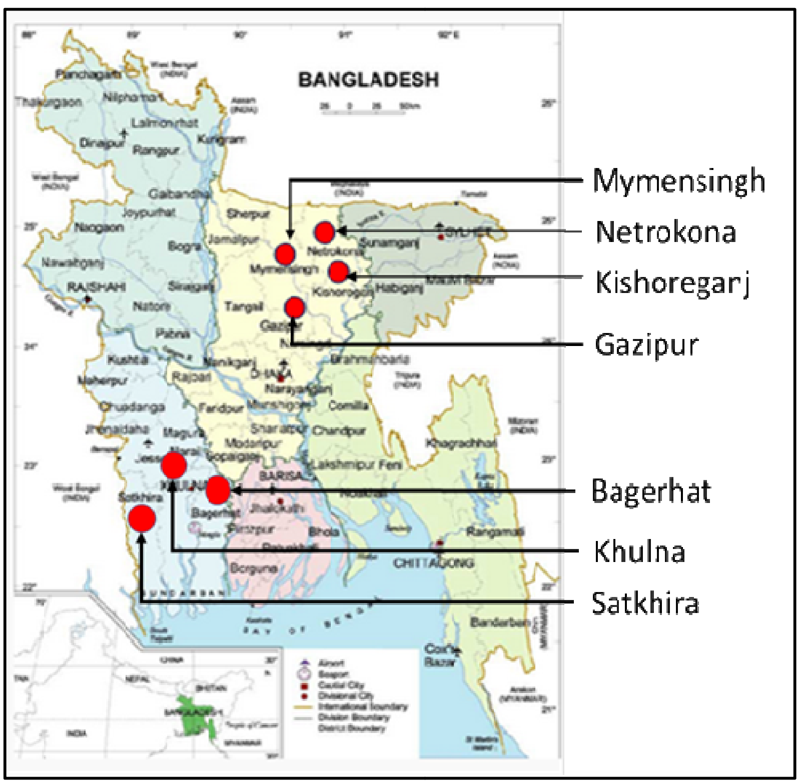


Fig. 1. Red circle of the map showing the infected fish sample collected areas of different districts of Bangladesh.

10.6. Materials used

10.6.1 Sample collection materials

Apron, mask, sterile hand gloves, markers pen, field notebook, sterile zipper packs, icebox, 70% ethanol and 0.1% peptone water were used for sample collection. After collection, the samples were labeled properly and brought into the laboratory as soon as possible.

10.6.2. Glass wares and other appliances

The glass wares and appliances used during the study period were as follows: scalpel, forceps, scissors, tray, petridishes, test tubes, conical flask (500, 1000, 3000 and 5000 ml capacities), pipette, micro pipettes (100-1000µl, 20-200µl, 2-20 µl , 0.5-10µl), glass slides, hanging drop slides, glass rod spreader, test tube racks, PCR tubes, micropipette tips, Micro-oven, water bath, bacteriological incubator, refrigerator, autoclave machine, hot air oven, centrifuge machine, Bio-safety cabinet, Gas burner, ice boxes, electronic balance, disposable plastic syringe and needle, compound microscope, Thermo-cycler, UV-transilluminator, High speed and Eppendorf refrigerated centrifuge machines, normal refrigerator, -20^0C and -80^0C freezer.

10.6.3. Chemicals and reagents

Phosphate buffered saline (PBS) solution, reagents for Gram's staining (methylene blue, Crystal violet, Gram's iodine, Acetone, Ethyl alcohol and Safranin), immersion oil, 3% Hydrogen peroxide, Rabbit plasma, 50% buffered glycerin, Alcohol, Kovac's indole reagent (4-dimethylamino-benzaldehyde, concentrated HCl), Aluminum hydroxide gel (adjuvant), a non-mineral oil adjuvant made of vegetable oil (Montanide), Fruend's complete adjuvant, 37% Formaldehyde, Potassium permanganate, and other common laboratory chemicals and reagents , distilled water, ELISA kit, PCR reaction mixture, genus and species specific primers, enzymes, Agarose NA gel, Et-bromide/ Midori Green, TAE buffer, 6 X dye, 100 bp and 1 kb DNA marker were used for in this research.

10.7. Culture media

10.7.1.Semi-solid culture media

Plate Count Agar (PCA), Tryptic Soy agar (TSA), Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS), Blood Agar (BA), Brilliant Green Bile Salts-Starch (BGBSS), Ecosan-Xylose-Ampicillin (EXA), Dextrin Fuchsin-Agar (DFS), Inositol-Brilliant green-Bile Salts (IBBS), Pril-xylose-agar (PXA), Xylose-Desoxycholate-Citrate Agar (XDCA), Rogol's medium (ROG) andMueller-Hinton agar media were used for bacteriological analysis as solid culture media for this study.

10.7.2. Liquid culture media

The liquid media used in the study were nutrient broth (NB), Tryptic Soy Broth (TSB), Brain Heart Infusion Broth (BHI) with 2% laboratory NaCl, Methyl-Red and Voges-Proskauer Broth (MR-VP), alkaline peptone water and sugar media.

10.8. Collection of dead/moribund fishes manifested by the various lesions and transportation of dead fish samples

Dead/moribund Tilapia, V. Koi, Shing, Magur, Pangas and Shrimp fishes were collected from 40 ponds and ghers of Mymensingh, Gazipur, Netrokona, Kishoreganj, Bagerhat, Khulna and Satkhira districts of 15 upazilla during several outbreaks starting from April-June of the year 2017, 2018 and 2019.

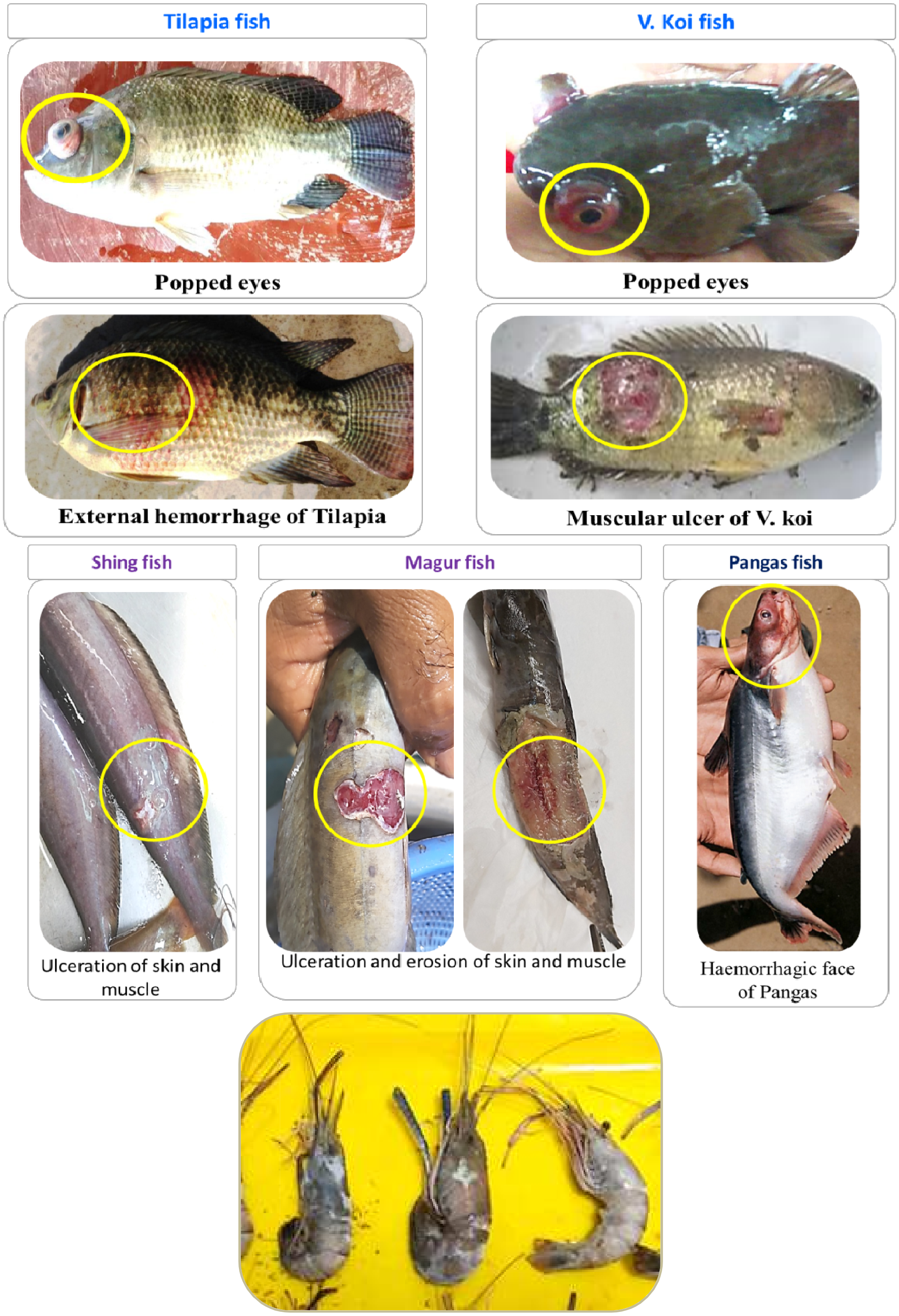
The fish samples were collected from affected ponds showing exophthalmia (popped eyes), ulcerative and hemorrhagic syndromes of Shing, Magur, Pangas, Koi, Tilapia and Early Mortality Syndrome (EMS) in Shrimp, loss of appetite, sluggish movement and swimming close to the surface of the water, lethargic, no escape reflex and erratic swimming. Naturally infected fishes were collected into sterile separate zipper plastic bags and transported to the laboratory of Fish Health Management, Freshwater Station, Bangladesh Fisheries Research Institute, Mymensingh maintaining cool chain for the isolation of bacteria and viruses.

Plate 2. Collection of infected fish samples.

Plate 3. Collection of diseased captured fishes from the affected ponds.

Plate 4. Moribund/death fishes at infected cultured ponds.

Plate 5. Transporting of death fishes to the lab. by maintaining cool chain.



Diseased Shrimp
(***Black discoloration of the body and softness of the cuticle of shrimp***)
Plate 6. Dead/moribund fishes manifested by the various lesions.

10.9. Species wise number of collected fishes from the affected ponds and gher

A total of 300 fish samples of which Tilapia (n= 60, bwt= 160±10gm) and V. Koi (n= 40, bwt= 120±10gm) Shing (n= 60, bwt= 90±5gm), Magur (n= 60, bwt= 110±10gm), Pangas (n= 30, bwt = 250±10gm) and Shrimp (n= 50, bwt= 50±5gm)were collected from 40 affected ponds of 15 upazila from 7 districts.

10.10. Selection of organs of the dead fishes for the isolation of bacteria and viruses

Bacteria were isolated from diseased or moribund Tilapia, V. Koi, Shing, Magur, Pangas and Shrimp by using the organs like liver, kidney, brain, spleenand skin tissue by a fume sterile loop method.

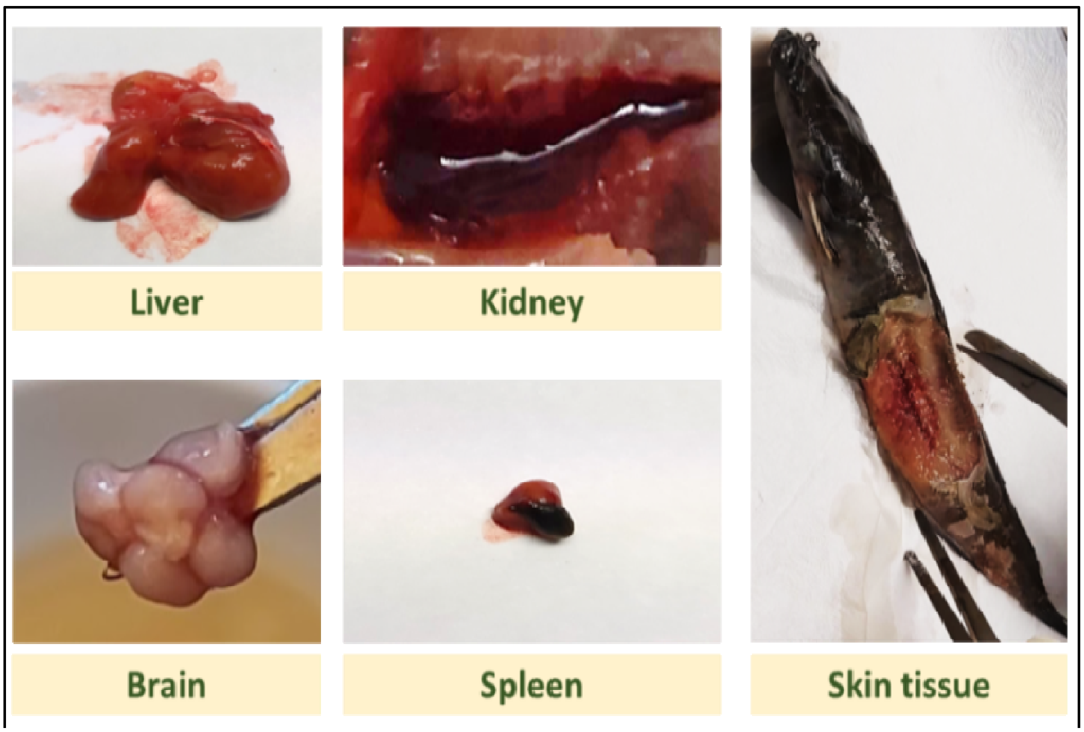


Plate 7. Various organs of dead fishes for the isolation of the bacteria.

10.11. Number of collected organs sample from different fishes

For the isolation, identification, molecular detection of bacteria and viruses the organs and skin tissue from Tilapia, Koi, Shing, Magur, Pangas and Shrimp samples were collected from the affected ponds and gher having mortality around 80-90%.

Table 1. List of collected organ samples fromTilapia, Koi, Shing, Magur, Pangas and shrimp fishes

Types and number of collected fishes from the affected ponds and gher	Collected organs and tissues from dead fishes	Number of the organs and skin tissue samples
Tilapia (n= 60), Koi (n= 40)	Brain, liver, kidney and spleen	400
Shing (n= 60), Magur(n= 60), Pangas (n= 30)	Liver, kidney, spleen and skin tissue	600
Shrimp (n= 50)	Brain and liver	100
Total sample		**1100**

10.12. Isolation and identification of pathogenic bacteria

The disease producing pathogenic bacteria were primarily isolated by BFRI (*Component 1*) following standard methods as described in the OIE Manual, 2017. The biological and molecular characterization of the isolated pathogenic bacteria (*Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus*) from Tilapia, V. Koi, Shing, Magur, Pangas and Shrimp were performed following the methods stated in the "The Shorter Bergey's Manual of Determinative Bacteriology (Holt, 1977) at the Department of Microbiology and Hygenie, BAU.

Isolation of the pathogenic bacteria from the fishes showed ulcerative and hemorrhagic syndromes (Tilapia, Koi, Shing, Magur and Pangas) and Early Mortality Syndrome (EMS) in Shrimp. Four important species of deadly bacteria *(Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus*) was isolated from the above mentioned five different species of fin fishes. Standard Operating Procedure (SOP) was followed for the biological and molecular characterization of the pathogenic bacteria from Tilapia, Koi, Shing, Magur, Pangas and Shrimp from different outbreaks of seven different districts of Bangladesh.

10.13. Primary isolation of pathogenic bacteria using selective media

TSA agar was used as a selective media for the observation of the colony morphology of *S. agalactiae* and TCBS ager was used as selective media for the observation of the colony morphology of *Aeromonas hydrophila* and *Aeromonas veronii.* The modified TCBS agar (with 2% NaCl) was used as a selective media for the observation of the colony morphology of *Vibrio parahaemolyticus* on TCBS agar.

Plate 8. Bacterial culture at disease laboratory.

10.14. Determination of colony morphology and staining characteristic of the isolated bacteria from different organs and tissues of dead fishes

A loopful cultured broth and individual colony from the 24 hours of incubated each sample (liver, kidney, brain and skin tissue) was stained by Gram's staining method and observed under 100X objectives for the visualization of the shape, arrangement and staining characteristic of the isolated bacteria.

10.15. Total Viable Count (TVC) and Total Specific Bacterial Count (TSBC)

Total Viable Count (TVC) was calculated from liver, kidney and brain tissue from naturally infected dead fishes. Total *Streptococcus* Count, Total *Aeromonas* Count and Total *Vibrio* count were calculated from different organs of naturally infected dead Tilapia, V. Koi, Singh, Magur, Pangas and Shrimp fishes

10.16. Biochemical tests conducted for the identification of the isolated bacteria

Catalase, Oxidase, Indole test, Voges–Proskauer (VP) test, Methyl Red (MR) Test, Arginine Dihydrolase (ADH) test, Oxidative-Fermentative (OF) test, Glucose, Mannitol and Lactose test for *Streptococcus* spp. NO_3 to NO_2, D-Glucose (gas), Indole test, Lysine decarboxylase, 0% and 6% NaCl tolerance, Sucrose, D-Manitol, L-Arabinose, Salicin and Manose test for *Aeromonas* spp. whereas Oxidase, D–Glucose (gas), Lactose, Sucrose, 0% and 6% NaCl tolerance, Sucrose, Inositol, Lysine decarboxylase, Arginine dihydrolase and Ornithine decarboxylase test for *Vibrio* spp. were conducted for the Biochemical tests for the isolated bacteria. All the above tests were jointly performed at *BFRI & BAU* laboratories.

10.17. Confirmation of the isolated bacteria by Polymerase Chain Reaction (PCR)

10.17.1. Materials required for extraction of bacterial DNA : Refrigerated micro centrifuge machine, vortexer, micropipette, tips for micropipette, nuclease free DEPC treated dH_2O, incubator, RNAse away solution, tube holding rack, 100 bp and 1 kb DNA marker, Bio-safety cabinet, Gas burner, Eppendrof tube, Ice powder and -20°C refrigerator.

10.17.2. Extraction of bacterial genomic DNA : The genomic DNA was extracted by the boiling method (Rawool *et al.*, 2007). A single colony of each bacterium was placed into 100 µl of distilled water in an Eppendorf tube, mixed well and then boiled for 10 minutes. After boiling, the tube was immediately placed into the crusted ice for cold shock for 5 minutes followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and used as template DNA for every species of bacteria.

10.17.3. Materials required for PCR : PCR master mix: A commercial PCR master mix (Taq 2X master mix, Biolab, USA) was used, specific primers (Table 2), Thermal Cycler, (Thermocycler, ASTEC< Japan), 2% agarose gel, gel casting tray with gel comb, TAE buffer, microwave oven, conical flask, 100 bp and 1kb DNA size marker, bromphenicol blue of loading buffer, ethidium bromide/ midori green (0.5µg/ml), distilled water, UV-trans illuminator

(Biometra, Germany), USB flash drive.For molecular detection of isolated bacteria, the primers used are presented in Table 2.

Table 2. List of gene specific primers sets used for the confirmation of isolated bacteria by PCR

Target genes	Species	Sequences	Amplicon size	References
16S rRNA –F	*Streptococcus agalactiae*	GAGTTTGATCATGGCTCAG	220 bp	Martinez *et al.,* 2001
16S rRNA –R		ACCAACATGTGTTAATTAC TC		
hyl –F	*A. hydrophila*	GGC CCG TGG CCC GAA GAT GCA GG	597 bp	Al-Fatlawy and Al-Ammar, 2013
hyl –R		CAG TCC CAC CCA CTT C		
16S rRNA –F	*A. veronii*	CCA GCA GCC GCG GTA ATA CG	300 bp	Al-Fatlawy and Al-Ammar, 2013
16S rRNA-R		TAC CAG GGT ATC TAA TCC		
flaE-F	*Vibrio parahaemolyticus*	GCAGCTGATCAAAACGTT GAGT	897 bp	Tarr*et al.*, 2007
flaE-R		ATT ATC GAT CGT GCC ACT CAC		

10.17.4. PCR amplification of bacteria : PCR reaction was performed for amplification of 16s rRNA gene of *Streptococcus agalactiae*, *hyl* gene of *Aeromonas hydrophila*, 16s rRNA gene of *Aeromonas veronii* and *flaE* gene of *Vibrio parahaemolyticus*in a gradient thermal cycler (Thermocycler, ASTEC, Japan) according to previously described protocols of Martinez *et al.,* (2001), Al-Fatlawy and Al-Ammar (2013) and Tarr *et al.,* (2007) respectively, with minor modification.

10.18. Pathogenicity test/biological characterization of the isolated bacteria: Determination of pathogenicity of the isolated bacteria in the healthy Tilapia, Koi, Shing, Magur and Pangas fishes by aquarium based experimentally induced infection

10.18.1.Selection and management of finfishes for experimental induced infection : Each of one hundred and twenty adult Tilapia (average bwt. 180g), V. Koi (average bwt. 135g) Shing (average bwt. 110g), Magur (average bwt. 160g) and Pangas (average bwt. 250g) were obtained from different commercial ponds. Fishes were randomly collected and performing microbiological examination at BFRI disease lab to confirm that they have no disease or any abnormality. The fishes were maintained in separate glass aquarium (50 liter) for 3 weeks and were kept at least 1 week for adaptation to the environment. During the acclimatization period, the fish were fed twice daily with commercial fish feed at the rate of 3% of their body weight until completion the experiment. The fishes were starved for 24 hrs before introduction of infection. The water was constantly monitored and renewed with 50% fresh water daily. Water quality parameters were measured using YSI 85 (Temperature 29±1.2 °C, DO 5.8±1.2 mg/l, pH 7.2±0.3, and ammonia 0.3±0.1 mg/L) (Alsaid *et al.*, 2013). These conditions were kept constant during entire period of the experiment.

10.18.2. Preparation of bacterial inoculum : The isolated and purified stock of *S. agalactiae, A. hydrophilus, A. veronii*and *V. parahaemolyticus* were allowed to grow onto the BHIA at 30 ºC for 24 hrs *at BAU lab*. Several identical colonies of each bacterium were inoculated into 10 ml BHI broth for 24 hrs at 30 ºC. After incubation, the broth was centrifuged at 15,000 rpm for 15 minutes at 4 ºC and the pellet was obtained. The pelleted bacteria were washed with phosphate-buffered saline (PBS) (Alsaid *et al.*, 2014). The bacterial suspensions was then diluted with sterile saline solution (0.85%) to reach the concentration at $6.3x10^6$CFU/ml, $1.3x10^6$CFU/ml, $2.3x10^6$ CFU/ml and 8.6 $x10^6$CFU/ml for *S. agalactiae, A. hydrophilus, A. veronii*and *V. parahaemolyticus* respectively (Iregui *et al.* 2016; Pereira *et al.* 2010).

10.18.3. Design for experimental infection of fishes by each bacterial isolate : Each species of fishes were divided into four separate experimental groups. Three trial experiments were designed as 1st, 2nd and 3rd for each species of fishes. PBS mock control group of fish were kept with each intramuscular (IM) experimental trial group and each group comprised of ten fishes kept in 120 liter glass aquarium.

The I/M group was inoculated with 0.3 ml of $6.3x10^6$CFU/ml, $1.3x10^6$CFU/ml, $2.3x10^6$ CFU/ml and $8.6x10^6$CFU/ml for *S. agalactiae, A. hydrophilus, A. veronii*and *V. parahaemolyticus* respectively according to Iregui *et al.,* (2016) and the control group was injected with 0.3 ml of PBS as mock control. All groups of fishes were observed twice daily to record any clinical signs, abnormal behaviour or mortalities from the date of inoculation of bacteria untilday-14th of post-infection.

Plate 9 . Experimental infection of fish at Lab.

10.19. Re-isolation and identification of bacteria from the experimentally infected dead fishes

Tissue samples (liver, kidney and brain) were collected from experimentally induced infected Tilapia, V. Koi, Shing, Magur and Pangas fishes immediately after their death. Re-isolation of bacteria from dead fishes was done using different bacteriological media as used previously for field samples. Bacterial isolates from experimentally infected fishes were subjected to identification by using different biochemical tests and molecular assay (PCR).

10.20. Stock preparation of pure culture of pathogenic bacteria

A locally isolated bacterial species, *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii and Vibrio parahaemolyticus* were used in this study. The strains were isolated from different outbreak of diseased cultured Tilapia, V. Koi, Shing, Magur, Pangas and Shrimp fishes from 2018-2020 of different outbreak areas of Bangladesh. The stock was kept in buffered glycerol at –80 °C was thawed to room temperature overnight before use.

10.21. Development of fish vaccines

10.21.1. Large propagation of bacterial isolates (each of the four species of the bacterial isolates of fishes) for the development of vaccine : The fortified broths were prepared with liquid enriched nutrient medium for the large propagation of isolated bacteria. Propagation was done by inoculating 10 colonies of each bacteria into 1000 ml fortified Brain Heart Infusion Broth (BHIB) at 37ºC for 24-48 hrs (Filev& Kabaivanov., 1981). The large volumes of propagated bacteria were used for the development of an activated vaccine.

Plate 10. Large propagation of each bacteria.

10.21.2. Inactivation and concentration of bacteria : The fortified broth of bacteria were centrifuged at 5000 rpm for 15 min (4ºC) and the pellets of bacteria were obtained after removing supernatant broth and washed twice with phosphate-buffered saline (PBS) (Alsaid *et al.,* 2014). The final concentration was determined 2×10^{12} CFU/ml for *S. agalactiae*,1.3×10^{10} CFU/ml for *A. hydrophila,* 2.3×10^{9} CFU/ml for *A.veronii* and 8.6×10^{9} CFU/ml for *V. parahaemolyticus* by using10-fold serial dilution (Iregui *et al.*, 2016; Pereira *et al.,* 2010). The inactivation of bacteria with formalin for vaccine preparation was prepared according to the method of Firdaus-Nawi *et al.* (2013).

Plate 11. Precipitation and washing of bacteria by centrifugation and adding of formaldehyde.

In the present study, a final concentration of 2.5% formaldehyde was used for the inactivation of each bacteria. However, the initial concentration of formaldehyde used in this study was 0.5% and it was increased gradually until the bacteria were inactivated entirely for safety reasons. The formaldehyde was removed by washing the concentrated pellet of the inactivated bacteria three times with phosphate-buffered saline (PBS) and then antibiotic (Ceftriaxone 10 mg/ml) was added. Inactivation of bacteria was confirmed by inoculating the cultured inactivated suspension into enriched nutrient media and observed for 24 h at 37°C for the appearance of any colony.

10.21.3. Measurement of formalin residue after final washing in the supernatant of the inactivated bacterial pellet washed with 1 X PBS : Formalin detection kit was used for the measurement of residue of formalin in the supernatant of final washing of inactivated bacterial pellet with PBS. This kitused here was developed by Bangladesh Council of Scientific and Industrial Research (BCSIR).

Plate 12. Formalin detection test kits.

10.21.4. Sterility test of formaldehyde inactivated bacteria : Sterility and safety of the newly developed fish vaccines were performed as per the OIE manual, 2019. The formalin-inactivated and antibiotic-treated bacteria inoculated into nutrient agar to observe the growth of any bacterial colony after incubation at 37ºC for 24-48 hrs.

Plate 13. Sterility test of each bacteria.

10.21.5. Mixing of bacterial antigen with adjuvant : In this study, a non-mineral oil adjuvant made of vegetable oil (MONTANIDE™ IMS-1312, SEPPIC, France) was used to improve the vaccine antigenicity and stability to fluid vaccines (Stone, 1993). Montanide was mixed with an inactivated antigen at the ratio of 1:1 for 10-20 minutes to obtain the expected safety and efficacy balance.

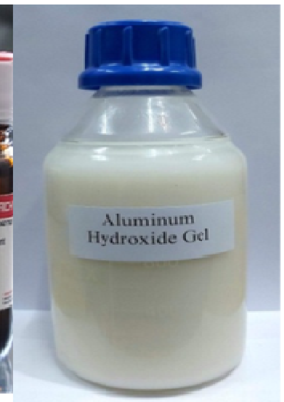


Plate 14. MONTANIDE™ IMS

Plate15. Fruend's complete adjuvant.

Plate16. Aluminium hydroxide.

10.22. Determination of efficacy of the newly developed fish vaccines by immunization and challenge experiment in healthy fishes at BFRI, Mymensingh

A total of five types of vaccine, two mono-valent (*S. agalactiae* and *A. veronii*), one bi-valent (*A. hydrophila+A. veronii*) and two tri-valent (*S. agalactiae+A. hydrophila+A. veronii*; *A. hydrophila+A. veronii+V. parahaemolyticus*) were prepared. The efficacy trials of these vaccines were applied intra-muscularly (I/M) doses of 0.1, 0.3 and 0.5 ml/fish.

10.23. Field Trial of experimentally developed vaccine

Two fish farms were selected for the vaccination programme. One is in MA Fisheries Douhakhola, Gouripur, Mymensingh and another is The MO Fisheries, which is in Trishal,Mymensigh. Magur, Shing and koi were selected from the MA Fisheries and Tilapia was chosen from MO hatcheries for vaccination process. The details of vaccination programme were described in the following Table 3. These vaccination processes were done two times mainly two consecutive breeding season on those farms.

Table 3. Details of Vaccination process

Place	Species	Vaccine	Dose
Douhakhola	Magur (10♀ + 5♂)	*Aeromonas hydrophila* *Aeromonas veronii* *Vibiro parahaemolyticus*	0.3 ml
	Magur (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas veronii* *Streptococcous agalactiae*	0.3 ml
	Koi (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas veronii* *Vibiro parahaemolyticus*	0.3 ml
	Koi (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas veronii* *Streptococcous agalactiae*	0.3 ml
	Shing (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas veronii*	0.3 ml
	Shing	*Aeromonas hydrophila*	0.3 ml
	Shing	*Aeromonas vironi*	0.3 ml
Trishal	Tilapia (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi* *Streptococcous agalactiae*	0.3 ml

Plate 17. Feed preparation and vaccinated Tilapia fry.

10.24. Experimental design for active immunization of healthy adult fin fishes

A total of 80 healthy Tilapia (180±5 g), V. Koi (120±5 g), Shing (110±5g) and Magur (150±5g) fishes were collected and equally divided into each four groups (IM, IP, oral and control groups). Ten fishes were taken into each of 8 glass aquarium for each experimental group. The fishes were maintained in a separate glass aquarium (120 litres), maintaining an average water temperature of (27.73±2.45 °C), dissolved oxygen (6.97±2.43 mg/l), pH (7.65±1.45) and ammonia (0.01±0.00 mg/l) ranged on acceptable levels overall the experimental periods (Monir *et al.*, 2020). The fishes were fed with commercial fish feed (Mega Feed, Bangladesh) at the rate of 5% of their body weight twice daily at 8 am and 3 pm.

10.24.1. Number of trials and treatment of experiment : The experiment consisted of two treatments and one controls with three replicates.

In treatment 1, Tilapia, V. Koi, Shing and Magur fishes from each group were vaccinated IP, IM and orally with 0.3 ml of vaccine (2×10^7 CFU/ml) on day 0 and 1st booster was done at day 14.A fresh inoculum containing live virulent cells of locally isolated *S. agalactiae* bacteria at 0.1 ml/dose of 1×10^7 CFU/ml was used for challenge experiment in oral, IP, IM and control group for each group of fishes on day 28.

In treatment 2, Tilapia, V. Koi, Shing and Magur fishes from each group were vaccinated IP, IM and orally with 0.3 ml of vaccine (2×10^7 CFU/ml) on day 0, 1st booster and 2nd booster was done at day 14 and 28, respectively. A fresh inoculum containing live virulent cells of locally isolated *S. agalactiae* bacteria at 0.1 ml/dose of 1×10^7 CFU/ml was used for challenge experiment in oral, IP, IM and control group for both Tilapia and V. Koi fishes on day 42.

After challenged, all fish were closely observed at hourly intervals for physical changes and behavioural. At the end of day-14 post-challenge, all surviving Tilapia, V. Koi, Shing and Magur fishes were subjected to post-mortem examination before samples of liver, kidney and brain were collected for bacterial isolation. The level of protection or Relative Percentage Survival (RPS) value was then determined (Amend, 1981). The blood samples were collected from each four groups on 7 and 21 days of post vaccination to determine the haemato-immune response.

10.24.2. Collection of blood sample from vaccinated fishes of each treatment : The blood samples were collected from four groups (three routes for vaccinated and one control) on 7, 21, and 35 days of post -vaccination (dpv). The blood sample was collected through the caudal veins from 5 fishes of each group and kept in two different type of tubes, one EDTA-containing tubes where another one without EDTA-containing tubes. The blood samples of EDTA-containing tubes were immediately transferred to the laboratory for haematological parameters analysis but without EDTA-containing samples were used to assess serum antibody level.

10.24.3. Haematological assays of the vaccinated and non-vaccinated fishes : The EDTA-containing tubes of blood samples were used to determine the leukocyte, lymphocytes, monocytes and granulocytes count using modified wright staining method

10.24.4. Detection of antibody level (serum IgM) of the vaccinated fishes by ELISA : Serum antibody of each species of vaccinated fish was determined by indirect ELISA test using specific bacterial antigens coated plates provided by Nordic, Netherland. The titre of serum of each time point for each species of fish was determined against IgM of vaccinated fishes by using ELISA reader at 450 nm.

10.24.5. Determination cut-off value by ELISA : The cut-off value is the highest possible true-positive rate (Holten *et al.*, 2012) used as an indication of protection. It was determined by performing ELISA on 200 serum samples (Tilapia= 100 and V. Koi= 100) collected from non-immunized Tilapia and V. Koi farms with no history of Streptococcosis infection and did not practice vaccination against the target organism. After excluding the blank wells, the average OD value was times 2 to get the cut-off value of 0.2 OD.

10.25. Active followed by passive immunization of brood stock Tilapia, Koi, Shing and Magur fishes with inactivated bacterial vaccines

10.25.1. Stage 1: Immunization of brood stock fishes : Forty-eight of each Tilapia (280±5 g), V. Koi (220±5 g), Shing (210±5g)and Magur (250±5g) brood stock fishes were divided into four groups namely intra-muscular (IM), intra-peritoneal (IP), oral and control with nine females and three males in each group in a container. The brood stock fishes were immunized by IM, IP and orally; meanwhile the unvaccinated brood stock fishes were used as control.

10.25.2. Rearing of fertilized eggs and larvae of fishes at hatchery level : The fertilized eggs from Tilapia, V. Koi, Shing and Magur fishes were obtained from the brood stocks and they were kept incubated until hatching. The fish larvae were fed with commercially available powder feed ad libitum after 7 days of hatching.

10.25.3. Challenge test of reared larvae from vaccinated fishes : The efficacy of the *S. agalactiae* vaccine of IM, IP and orally immunized and non- immunized (control) Tilapia, V. Koi, Shing and Magur fishes was obtained by conducting the test of immersion challenge to larvae produced from brood stock immunization. Four groups of larvae from each group of fish were immerged infected with dilution (10^7 CFU/ml) for 30 min. Challenge test of larvae was carried out at 5, 10, 15 and 20 days after hatching. The mortality was observed for 12 days and dead fish larvae were confirmed for *S. agalactiae.* The cumulative mortality from immunized and non- immunized fish for each experiment was determined over a 12-day period. The efficacy of the vaccine was calculated as Relative Percent Survival (RPS) according to the method of Amend (1981).

10.25.4. Preparation of serum from immunized brood stock : The blood sampling of brood stock fish was carried out on the 7^{th} and 14^{th} days of vaccination. The blood samples were collected from the heart of brood stock Tilapia, Koi, Shing and Magur fishes. The serum was collected from blood by centrifugation at 5,000 rpm for 10 min at 4°C. The serum was separated and taken into a new micro tube and stored at -20°C, which could be used for antibody determination (Sukenda *et al.*, 2017).

10.25.5. Preparation of tissue homogenate from eggs and larvae of immunized fishes : The sampling method of eggs and larvae homogenates was carried out according to Hanif *et al.* (2004). The egg samples were collected from four (IM, IP, oral and control) brood stock of the immunization treatment groups. The larvae were collected at 5, 10, 15 and 20 days after hatching. Each egg and larvae sample were homogenized in PBS-T (PBS + 0.05% Tween-20) solution at a ratio of 1:4. Besides, it was centrifuged at 5000 rpm for 10 min. All samples were stored at –20ºC for further use to determine the antibody level.

10.25.6. Determination of antibody level (IgM) in the immunized brood stock, their eggs and larvae by ELISA : The antibody level of brood stock, eggs and larvae were measured using the indirect Enzyme-Linked Immunosorbent Assay (ELISA). The antibody level of the brood stock Tilapia, V.Koi, Shing and Magur fishes were measured on the 7^{th} and 14^{th} days of post-vaccination, while that of the larvae was measured on the 5^{th}, 10^{th}, 15^{th} and 20^{th} days after hatching. The antibody titre was measured using an ELISA method described by Nurani *et al.*, (2020).

10.26. Stage 2: Immunization of fish larvae through feeding of immunized hen egg yolk

10.26.1. Active immunization of healthy laying hen immunized with fish vaccine : Immunization of four laying hens using each type of fish vaccine has been started in the small animal laboratory, Dept. of Microbiology and Hygiene, BAU for the production of immunized hen eggs. Fry from the vaccinated brood fishes of each species will be fed with the egg yolk of the immunized hen eggs from day 3 to day 5 from 10 to 13 July, 2020 a view to transfer the immunoglobulins (IgM and IgY) raised against the antigens of each bacteria used in the vaccines. 25-week-old Bovans Brown (*Gallus domesticus*) laying hen were immunized S/C with 1 ml of formalin killed *S. agalactia* with montanide adjuvant with 1 ml booster injection alternated between the left and right side of the thigh at 27, 29, 31, 35 and 40 weeks (Lee *et al.*, 2000). Blood and eggs of immunized chicken were collected while the laying birds reached the age of 28 weeks. Every week, 3 ml of blood was taken from each laying bird and eggs from inoculated chickens were taken every day during the immunization period.

10.26.2. Preparation of purified hen egg yolk powder containing IgY in the eggs yolk of hen vaccinated with fish vaccines :

Egg yolk powder : After collection of egg, the egg yolk was separated from eggs, yellow egg yolk powder was obtained directly by freeze-drying.

Purified IgY powder : IgY was purified from the water-soluble fraction by a combination of several purification techniques including two-step salt precipitation according to Lu *et al.*, (2009).

10.26.3. Preparation of fish larval feed with hen eggs yolk

All the necessary ingredients (egg yolk powder, skimmed milk and flour) of diet were purchased from the market to prepare the feed used in the experiment. There were two types of feeding phase (cistern phase and nursing phase) used in this study. In cistern phase, the egg yolk powder (50% w/w), skimmed milk (25% w/w) and flour (25% w/w) were mixed properly with blander. However, in nursing phase the egg yolk powder (50% w/w) were mixed properly with commercial powdered feed (50% w/w, Mega feed). The egg yolk powder was contained IgY (1% w/w). The feed added with IgY dried under a stream of ambient temperature air.

10.26.4. Oral administration of IgY

The fish larvae of Tilapia, V. Koi, Shing and Magur fishes were fed three times daily at 15% of body weight with a newly developed feed-based vaccine up to 5 weeks.The control group was given feed added nonspecific IgY.

10.26.5. Challenge test of fish larvae after egg yolk feeding

The efficacy of the *S. agalactiae* vaccine of orally immunized and non- immunized (control) Tilapia, V. Koi, Shing and Magurfish'slarvae were found by conducting the test of immersion challenge. Four groups (7 days feeding, 14 days feeding 21 days feeding and control group) of larvae were immerged and infected with dilution (10^7 CFU/ml) for 30 min. Challenge test of larvae was carried out at 7, 14 and 21 days after fed with feed-based vaccine and also for control groups. The mortality was observed for 12 days and dead fish larvae were confirmed for *S. agalactiae.* The cumulative mortality from immunized and non- immunized fish larvae for each experiment was determined over a 12-day period. The efficacy of the vaccine was calculated as Relative Percent Survival (RPS) according to the method of Amend (1981).

11. Results and discussion (*Combined*)

11.1 Results of baseline survey on diseases of cultured fin fishes and shrimp

Global fish production peaked at about 171 million tons in 2016, with aquaculture representing 47% of the total and 53% if non-food uses (including reduction to fishmeal and fish oil) are excluded. Aquaculture continues to grow faster than other major food production sectors although it no longer enjoys the high annual growth rates of the 1980s and 1990s (11.3 and 10.0%, excluding aquatic plants). Average annual growth declined to 5.8 % during the period 2000–2016, although double-digit growth still occurred in a small number of individual countries, particularly in Africa from 2006 to 2010 (FAO, 2018).

Bangladesh is one of the most leading countries for Aquaculture in the world. Due to intensification of culture system, disease is now becoming a major threat which ultimately reduces the production. By the increasing intensification of fish production and lack of health management measures have led to many disease problems of bacterial, viral, fungal and parasitic origin. Fishes have been suffering from many diseases such as Epizootic Ulcerative Syndrome (EUS), tail and fin rot, fungal, parasitic and bacterial infections (Chowdhury *et al.*1999). In most cases haemorrhages, septicaemia, lesions, gill damage are the common symptoms of the diseased fish (Chowdhury 1993 & 1998). Pathogens, excessive high stocking density, higher input and inadequate husbandry practices accelerate diseases in the fish farms. Some diseases have caused serious damage, not only the livelihood of fish farmers, but also to the future development of the fish industry. From the annual report of Bangladesh Fisheries Research Institute following table was prepared to realize the past situation of diseases that occurred in different culture region in Bangladesh.

Table 4. Baseline survey data of disease occurrence during the year 2014-2018

Species Name	Culture Area	Causative Agent	% Mortality	Year
Tilapia (*Oreochromis niloticus*)	Mymensingh, Netrokona, Gazipur, Chandpur	*Streptococcus agalactiae*	60-70	2015-2018
		Virus	80-90	2017-2018
Vietnamese Koi (*Anabas testudineus*)	Mymensingh, Netrokona, Narsingdi	*Streptococcus agalactiae*	50-65	2015-2017

Species Name	Culture Area	Causative Agent	% Mortality	Year
Shing (*Heteropneustes fossilis*)	Mymensingh, Netrokona, Kishoreganj	*Aeromonas* sp. *Pseudomonas* sp.	30-40	2015-2017
		Virus	80-100	2014-2017
Magur (*Clarias batrachus*)	Mymensingh, Netrokona, Kishoreganj	*Aeromonas* sp. *Pseudomonas* sp.	40-50	2015-2018
		Virus	70-80	2014-2017
Pangas (*Pangasius hypophthalmus*)	Mymensingh, Gazipur,	*Aeromonas* sp. *Pseudomonas* sp.	40-60	2015-2016
Shrimp (*penaeus monodon*)	Bagerhat, Satkhira, Khulna	*Vibrio parahaemolyticus*	100	2015-2017
		WSSV	80-100	2015-2017

Summary of baseline study

- Huge number of fin fishes (Tilapia, V. Koi, Shing, Magur and Pangas) and Shrimp were found to be dead under culture condition in the stated districts.
- Fish farmers of many cultured ponds of the affected areas failed to control their fish mortality adapting all sorts of conventional and modern techniques, such as alteration of water, collection and toughing of dead fishes from the affected ponds, administering antibiotics and probiotics etc.
- Due to high mortality of fin fishes and shrimp in the cultured ponds and gher many farmers became looser financially

11.2 Water quality

11.2.1 Physico-chemical parameters of fish culture ponds

Water quality is the most important limiting factor in rearing of fish and directly affects feed efficiency, growth rate, the fish's health and survivability (Svobodová, 1993). Water quality parameters tolerance limits for fish vary from species to species through which they can survive, grow, and reproduce. Increasing incidence of fish disease attributed to poor water quality has forced many farmers to abandon aquaculture.

11.2.2 Water temperature

For optimal growth and survival, fish require a specific temperature (Brett *et al*., 1969; Gadomski and Caddell, 1991). These may differ depending on age and size, as many species' juveniles prefer warmer temperatures than adults (McCauley and Huggins, 1979; Pedersen and Jobling, 1989). The results of this study reveal that the optimal temperature range for fish (V. Koi, Shing, and Tilapia) is comparable, but the obtained value varies by species and period. Furthermore, temperature can fluctuate somewhat (increase or decrease) depending on a variety of factors such as sun radiation, air temperature, and so on. The amount of dissolved oxygen that a body of water can contain, the pace of decomposition, and photosynthesis are all influenced by the temperature of the water, which has an impact on oxygen demand in pond systems and ammonia ionization (Colt and Tomasso, 2001).

When temperature increases, then metabolic activity of fish also increases which ultimately increased oxygen consumption rate. It has been calculated that raising the water temperature from 9°C to 15°C reduces the capacity of water to hold oxygen by 12.8% (Klontz 1993). In addition, increasing temperature increases the growth and infectiousness of many fish pathogens (Roberts,2012) and increases the toxicity of many dissolved contaminants (Wedemeyer, 1996).

11.2.3 Water pH

pH is the measure of the acidity of a solution of water. Most species can tolerate a pH between 6 and 9 fairly well, but they are usually stressed by pH outside this range resulting in less growth and greater susceptibility to disease. The optimum pH for most species is between 7 and 8.5. pH value in experimental fish (Tilapia, Koi, Shing, Magur) were within range which is a positive finding for fish health. Since, If the pH of water is too high or too low, the aquatic organisms living within it will die. pH can also affect the solubility and toxicity of chemicals and heavy metals in the water (EPA, 2012). Likewise, Lower concentration of pH increases the toxicities of hydrogen sulphide (H_2S), copper and other heavy metals to fish. Fishes are prone to attack of parasites and diseases in acidic waters. When pH rises over 11, the gills and lens and cornea of fish eyes are destroyed (Jhingran, 1988). As a result the fishes become weak and infected by parasites.

11.2.4 Dissolved Oxygen of water

Dissolved oxygen (DO) is the primary water quality consideration for any fish farmer. Oxygen passively diffuses into water from the atmosphere, and the maximum amount that will dissolve depends upon a number of variables including temperature, salinity and altitude. Fish extract oxygen from the water by passive diffusion through the gills. An adequate concentration of DO in the water is required to facilitate the passive diffusion down a concentration gradient from the water into the blood (Colt and Tomasso, 2001). In present conducted study, DO concentration range were vary from species to species which sometimes fall below requirement level that possess risk for fish. Most fish have some ability to cope with fluctuations in dissolved oxygen, but if severe hypoxia persists the fish will eventually die (Fitzgibbon *et al.,* 2007; Cook and Herbert, 2012).

If DO concentrations fall below the requirements of the fish, then fish cannot convert energy as efficiently into a usable form, resulting in reduced growth rate, food conversion efficiency and swimming ability (Jones, 1971). It has been reported that salmonids show a behavioural avoidance of low oxygen levels (Levy and Northcote, 1982) and there are observations that the distribution of fish changes, with fish moving towards the surface or water inflow where DO concentrations are higher (Wedemeyer, 1996). However, when DO approaches lethal levels effects such as anorexia, respiratory distress, tissue hypoxia, precede unconsciousness and death (Wedemeyer, 1996).The low feed intake and low growth observed in fish at low DO conditions were because fish appetite and digestibility was reduced (Tran *et al.*, 2008; Gan *et al.*, 2013). Thus, it could be concluded that high growth under normal DO conditions resulted mainly from better feed consumption and nutrient digestibility.

11.2.5 Un-ionized ammonia in water

Ammonia is the primary waste metabolite produced by fish from the catabolism of protein contained within the feed. The ammonia is excreted from the fish via the gills (Evans *et al.,* 2005). Ammonia can also come from the decomposition of uneaten food, although this is considered a relatively minor source (Hinshaw and Fornshell, 2002). The un-ionized ammonia (UIA-N) is the toxic form for the aquatic organisms. The NH+ 4 is considered non-toxic. The percentage of total ammonia (TAN) present as UIA-N can be calculated by the aqueous ammonia equilibrium, which is strongly dependent on the temperature, pH and salinity (Lemarié *et al.*, 2004).The results of this study showed that, ammonia concentrations were so much higher from permissible limits for all of these (Tilapia, Koi, Shing and Magur) species which causes serious health alteration of fish.

The sensitivity of fish to increased levels of ammonia is influenced by many other factors. In general terms, sensitivity to ammonia decreases with age (MacIntyre *et al.*, 2008). Interestingly, when the oxygen in the water is depleted through fish respiration, the sensitivity to ammonia decreases, especially when the water exchange is, also, reduced (Tudor *et al.*, 1994). In this case, as $C0_2$ increases, pH is lowered and this, in turn, reduces the relative quantity of UIA-N. On the other hand, when environmental hypoxia occurs due to other reasons, then reduced DO increases the sensitivity of fish to ammonia. Short term exposures of fish to high concentrations of ammonia result in increased ventilation rate, hyperexcitability, erratic swimming, loss of equilibrium, convulsions and death. The physico-chemical parameters were measured from infected farms at Mymensingh region by using a portable HACH kit (Model FF-2).

Table 5. Water quality parameter of infected Vietnamese koi farms (January-September 2019)

Parameters	Result			Suitable range
	Tarakanda	Muktagacha	Trishal	
Temp (^{0}C)	26-32	25-29	26-29.5	28-32
pH	6.5-9.0	8.5-9.5	6.5-8.0	7.5-8.5
Dissolved oxygen (mg/L)	2.2-5.8	2.0-4.5	2.0-5.5	5.0-10.0
Un-ionized ammonia (toxic) (mg/L)	0.04-2.40	0.6-3.0	0.31-2.5	0.00-0.02

Table 6. Water quality parameters of diseased Shing and Magur farms (January-September 2019)

Parameters	Result			Suitable range
	Tarakanda	Muktagacha	Gouripur	
Temp. (^{0}C)	24-35	25-34	24.5-32	28-32
pH	7.5-9.0	8.5-12	7.6-8.0	7.5-8.5
Dissolved oxygen (mg/L)	1.0-5.0	3.0-4.5	1.5-5.0	5.0-10.0
Un-ionized ammonia (toxic) (mg/L)	0.04-1.2	0.6-2.2	0.31-1.5	0.00-0.02

Table 7. Water quality parameters of diseased Tilapia and Pangas farms(January-September 2019)

Parameters	Result				Suitable range
	Bhaluka	Muktagacha	Trishal	Kaliakoir	
Temp. (^{0}C)	15-31	16-32	26-29.5	26-29.5	28-32
pH	6.2-9.5	6.5-9.0	7.6-8.0	7.6-8.0	7.5-8.5
Dissolved oxygen (mg/L)	3.2-5.5	2.0-5.0	2.0-5.0	2.0-5.0	5.0-10.0
Un-ionized ammonia (toxic) (mg/L)	0.04-2.40	0.4-2.0	0.31-2.0	0.31-0.02	0.00-0.02

Table 8. Water quality parameters of different shrimp ghers (January-September 2019)

Parameters	Result					Suitable range
	Gher I	Gher II	Gher III	Gher IV	Gher V	
Depth of the Gher(feet)	4	3.5	3.0	2.5	3.0	3.0
Temp. (^{0}C)	24-26	24-29	25-29	22-32	22-31	28-32
pH	7.5-8.5	7.5-8.0	7.6-8.0	6.5-8.0	6.0-8.0	7.5-8.5
Dissolved oxygen (mg/L)	6.05-7.0	4.5-5.8	5.0-6.0	2.0-5.0	3.0-5.0	5.0-10.0
Salinity	3-4	2.5-4.5	2-4	3-6	4-5.5	6.5-6.8
Un-ionized ammonia (mg/L)	0.04-0.24	0.6-0.9	0.31-0.4	0.02-0.5	0.02-1.0	0.00-0.02

11.3 Isolation and identification of emerging and existing pathogenic bacteria and viruses responsible for mass mortality of fin fishes and shrimp

Four different types of highly pathogenic bacteria species *(Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus*) have been isolated fromLiver, Brain, Kidney, Spleen and skin tissue of five different species of fin fishes (Tilapia, V. Koi, Shing, Magur and Pangas) and Shrimp. *Streptococcus agalactiae* has been isolated from Tilapia and V. Koi fishes. The *Aeromonas hydrophila*and *Aeromonas veronii* have been isolated from Shing, Magur and Pangas. Whereas *Vibrio parahaemolyticus* bacteria has been isolated from Shing, Magur and Shrimp. All the isolated species of bacteria of fishes were confirmed by PCR using genus specific primers in this study.

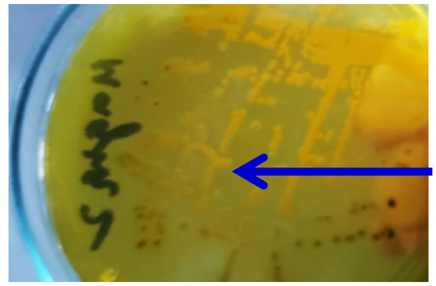
A total of 1100 samples,400 samples were isolated and characterized for *Streptococcus agalactiae* from Tilapia and V. Koi fishes, 368 samples were isolated and characterized for*Aeromonas hydrophila* and 232 samples for *Aeromonas veronii* from Shing, Magur and Pangas fishes. Whereas, 100 samples were isolated and characterized as *Vibrio parahaemolyticus* from Shing, Magur and Shrimp.

Results of isolation of pathogenic bacteria from different types of fin fishes of the study were similar with the results of Wang *et al*., (2013). They isolated and characterized the *Streptococcus agalactiae* from the kidney, liver and brain of all dead Tilapia fish and demonstrated that the isolated strain was virulent for the fish.The results of the present study agreed with the findings of Rahman *et al.* (2002). They identified and characterized the pathogenic *Aeromonas hydrophila* and *Aeromonas veronii* bacterial isolates associated with Epizootic Ulcerative Syndrome (EUS) in different fin fishes in Bangladesh. They were isolated 52 *Aeromonas* strains from EUS lesions in the fish. Sankar *et al.* (2012) identified and characterized the*Vibrio parahaemolyticus* from the diseased Shrimp fish and strongly recommended that the dominant *Vibrio* species might have acted as a secondary pathogen in the viral disease outbreak.

11.3.1 Identification of the isolated bacteria by cultural and morphological characteristic of the bacteria with Gram's staining

- Pen headed
- White opaque color
- Circular
- Entire raised edges
- Glistening colony

Plate 18. Colony morphology of *S. agalactiae*

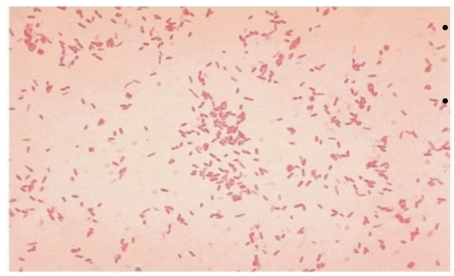


Yellow shin colony with diameter ranged from (2-3) mm

Plate 19. Colony morphology of *Aeromonas hydrophila* on TCBS ager

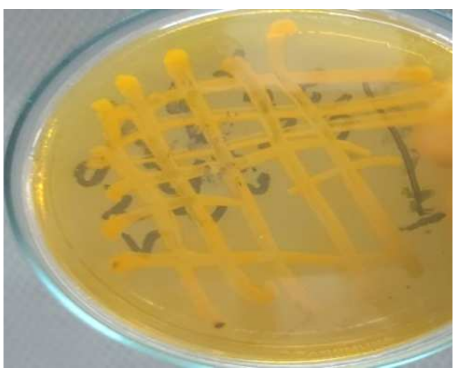
- Gram-positive
- paired and chain formed coccus

Plate 20. Morphological characteristic of *S. agalactiae* with Gram's staining



- Gram-negative, straight rods
- pairs with or without short

Plate 21. Morphological characteristic of *Aeromonas hydrophila* with Gram's staining

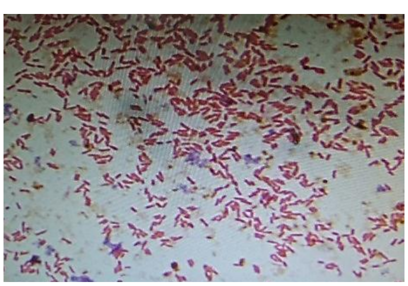


Yellow shiny colony

Plate 22. Colony morphology of *Aeromonas veronii*on TCBS agar

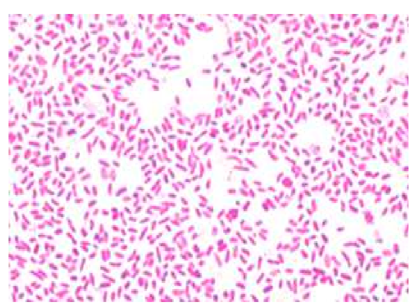
Greenish shiny colony

Plate 23. Colony morphology of *Vibrio parahaemolyticus* on TCBS agar



- Gram-negative straight rods appearing singly or in pairs with or without short chains

Plate 24. Morphological characteristic of *Aeromonas veronii* with Gram's staining



- Gram-negative curved rod-shaped

Plate 25. Morphological characteristic of *Vibrio parahaemolyticus* with Gram's staining

11.3.2. Isolation of Tilapia Lake Virus (TiLV)

A newly emerged highly pathogenic virus was isolated from Tilapia fishes form different outbreak areas of four different districts during 2018. The TiLV isolated from Tilapia was also re-isolated from experimentally induced infection in healthy Tilapia in the aquarium of BFRI, Mymensingh. The experimentally infected (through intra-peritoneal and intra-muscular route of infection) fishes revealed similar characteristic signs and symptoms with 36-60 hours of post infection of naturally infected TiLV fishes at aquarium-based infection. From the experimentally infected fishes similar RNA hemagglutinating viruses had been isolated using special techniques of fish virus isolation (both in-vivo and in-vitro system of propagation).

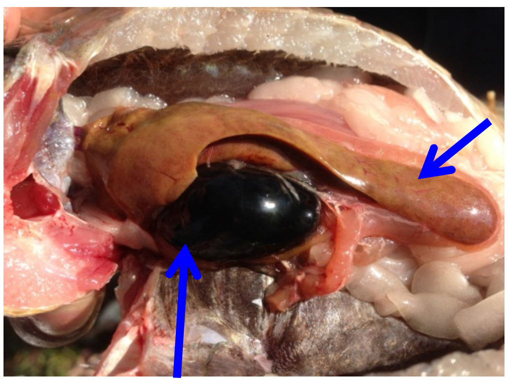


Plate 26. Post mortem lesion in the liver (hepatomegaly), and Gall bladder (extremely distended) of the TiLV affected Tilapia

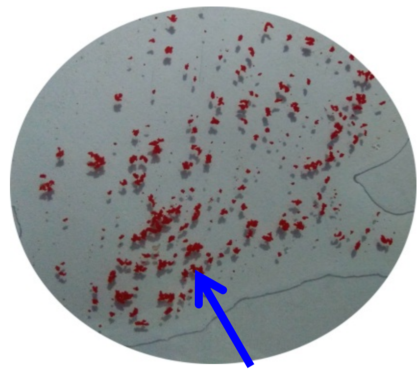


Plate 27. Hemagglutinating property of TiLV, showing clumping of cRBC

11.4. Total Viable Count (TVC) and Total Streptococcus Count (TSC) from different organs of naturally infected dead Tilapia and V. Koi fishes

Organ of the fishes	**Tilapia**		**V. Koi**	
	TVC (CFU/gm of sample)	TSC (CFU/gm of sample)	TVC (CFU/gm of sample)	TSC (CFU/gm of sample)
Liver	7.9×10^7	7.4×10^6	4.4×10^6	3.2×10^5
Kidney	6.6×10^7	5.5×10^5	4.1×10^5	2.2×10^4
Brain	5.9×10^4	2.8×10^4	8.4×10^2	3.5×10^2

11.5. Total Viable Count (TVC) and Total Aeromonas count from different organs of naturally infected dead Singh, Magur and Shrimp

Different organ of the fishes	Singh		Magur	
	TVC (CFU/gm of sample)	Total *A. hydrophila* count (CFU/gm of sample)	TVC (CFU/gm of sample)	Total *A. hydrophila* count (CFU/gm of sample)
Liver	1.3×10^{8}	4.2×10^{7}	3.4×10^{8}	2.2×10^{7}
Kidney	6.7×10^{7}	3.1×10^{6}	2.1×10^{6}	3.0×10^{6}
Brain	4.9×10^{4}	2.9×10^{4}	7.1×10^{2}	3.3×10^{2}

Different organ of the fishes	Pangas		Shrimp	
	TVC (CFU/gm of sample)	Total *A. veronii* count (CFU/gm of sample)	TVC (CFU/gm of sample)	Total *A. veronii* count (CFU/gm of sample)
Liver	6.1×10^{8}	6.4×10^{7}	3.4×10^{7}	2.2×10^{6}
Kidney	5.1×10^{7}	4.5×10^{5}	3.1×10^{5}	1.1×10^{4}
Brain	3.0×10^{4}	2.8×10^{4}	5.4×10^{2}	3.3×10^{2}

11.6. Total Viable Count (TVC) and Total Vibrio count from different organs of naturally infected dead Singh and Shrimp

Different organ of the fishes	Singh		Shrimp	
	TVC (CFU/gm of sample)	Total *Vibrio parahaemolyticus* count (CFU/gm of sample)	TVC (CFU/gm of sample)	Total *Vibrio parahaemolyticus* count (CFU/gm of sample)
Liver	6.7×10^{7}	6.3×10^{6}	4.2×10^{6}	2.3×10^{5}
Kidney	5.9×10^{7}	5.1×10^{5}	3.9×10^{5}	3.2×10^{4}
Brain	4.8×10^{4}	2.4×10^{4}	7.2×10^{2}	4.1×10^{2}

According to the findings, the isolated bacterial load in the liver of each fish was greater than the kidney and brain. Presence of bacteria in the brain of both species of fishes clearly indicates that the bacteria are highly pathogenic and possessed multi-organ invasive power.The results of the present study agreed with the findings of Wang *et al*., (2013). They found that the bacterial load in the liver of the diseased fish showed higher compared to that of kidney, spleen and brain.

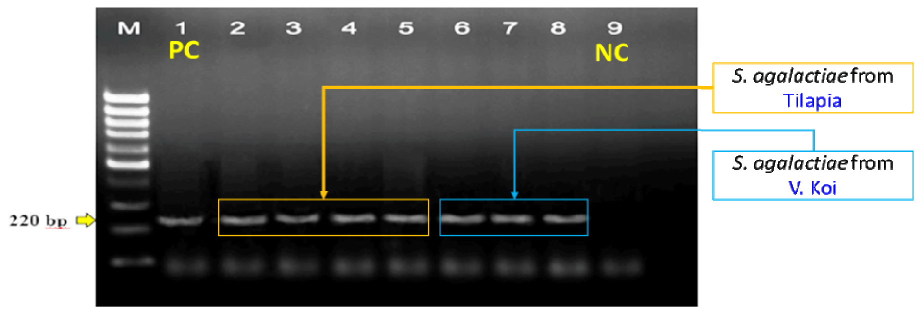
11.7 Identification of bacteria by various biochemical tests

Table 9. Identification of *S. agalactiae, A. hydrophila, A. veronii* and *V. parahaemolyticus* bacteria by various biochemical tests

Biochemical tests	**Results**			
	S. agalactiae	***A. hydrophila***	***A. veronii***	***V. parahaemolyticus***
Catalase	-	X	X	X
Oxidase	-	+	+	+
Indole	-	+	+	+
VP (Voges–Proskauer) test	+	+	+	-
MR (Methyl Red) test	+	+	+	+
NO_3 to NO_2	X	+	+	+
ADH (Arginine Dihydrolase) test	+	+	+	-
Oxidative-Fermentative (O/F) test	F	+	+	-/+
NaCl tolerance 0%	+	+	+	-
NaCl tolerance 1%	+	+	+	+
NaCl tolerance 3%	-	+	+	+
NaCl tolerance 5%	-	-	-	+
NaCl tolerance 6%	-	-	-	+
Urease	-	-	-	+
Glucose (Gas production)	+	+	+	-
Inositol	-	-	-	-
Mannitol	-	+	+	+
Lactose	-	-	-	-
Sucrose	+	+	+	-
Maltose	+	+	+	+
Mannose	+	+	+	+
Lysine decarboxylase	X	+	-	+
L-Arabinose	-	+	-	+
CAMP test	+	X	X	X
Hemolysis	β-hemolysis	β-hemolysis	β-hemolysis	β-hemolysis

+ indicates positive result, F indicates fermentative, - indicates negative result and X indicates tests not done

11.8 PCR result of S. agalactiae, A. hydrophila, A. veronii and Vibrio parahaemolyticus isolated from the field samples of Tilapia, V. Koi, Shing, Magur, Pangas and Shrimp



M	:	100 bp DNA ladder
Lane 1	:	Positive control
Lane 2-5	:	Positive isolates of *S. agalactiae* from Tilapia
Lane 6-8	:	Positive isolates of *S. agalactiae* from V. Koi
Lane-9	:	Negative control

Plate 28. PCR result of *S.agalactiae* isolated from the field samples from Tilapia and V. koi.

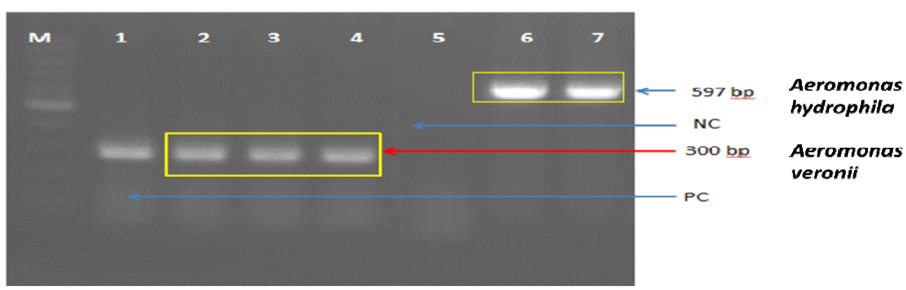


Image of 2% agarose gel electrophoresis showing the PCR amplification product of the 16SrRNA gene of *Aeromonas veronii*. M indicates 100 bp DNA ladder; Lane 1 is positive control; Lane 2-4 are positive isolates of *Aeromonas veronii*from Tilapia/Koi/ Magur/Shing; Lane 5 is negative control and Lane-6-7 are positive for *hyl* gene for *Aeromonashydrophila.*

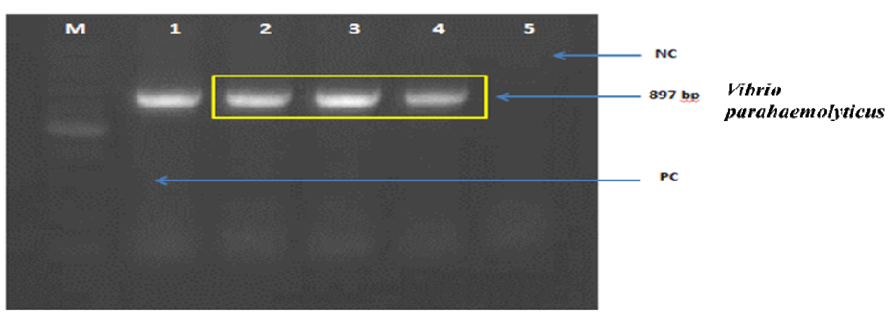


Plate 29. PCR result of *Vibrio parahaemolyticus* isolated from the field samples of Tilapia/Koi/Magur and Shing fishes.

Image of 2% agarose gel electrophoresis showing the PCR amplification product of the *Fla*E of *Vibrio parahaemolyticus* isolated from naturally infected diseased fishes. M indicates 100 bp DNA ladder; Lane 1 is positive control; Lane 2-4 are positive isolates of *Vibrio parahaemolyticus* Shing/Magur/ Shrimp; Lane 5 is negative.

11.9 Pathogenicity test results of the isolated bacteria for healthy fishes by aquarium based experimentally induced infection

Characteristic clinical signs (development of unilateral or bilateral exophthalmia, erratic swimming, pale gills, opaque cornea, external haemorrhages and ascites in abdominal cavities) were started manifesting by the both the Tilapia and V. Koi fishes of each (1st, 2nd and 3rd) trial group infected experimentally with *S. agalactiae.* Haemorrhagic and ulcerative symptoms were observed in each trial group infected experimentally with *A. hydrophilus, A. veronii*and *V. parahaemolyticus* I/M route of infection within day 1 of post infection and continue dying until day 6th of p.i.Whereas the fishes of mock (PBS) control groups did not die and even failed to show single signs of infection as manifested by bacteria infected fishes during entire period (14 days) of observation.

In this study, the fish mortality varied from 80-100%. The highest rate of mortality (100%) of the Tilapia and V.Koi fishes that were caused by *S. agalactiae*. The Shing and Magur fishes were found 100% mortality which were caused by *A. hydrophilus*and *A. veronii.* Whereas the highest rate of mortality (100%) found into the Pangas fish that was caused by *V. parahaemolyticus* in 1st to 3rd trial groups between days1-6th of post infection (Table 10-14). The remaining 20% survived fishes of each species of fishes those showed mild symptoms of diseases in experimental infection in three trial groups 1st- 3rdrecovered gradually within 7-14 days of post infection. The results of the present study agreed with the findings of Sarker and Faruk., (2016). The *A. hydrophila* bacteria found to be more virulent when administered through IM route resulted 70-80% mortality of the challenged fish.

11.10. Experimentally developed inactivated bacterial vaccines for fishes and their validation

Inactivated bacterial vaccines can be used in the healthy adult and brood (male and female) cultured fin fishes before starting their breeding seasons through injection. Same vaccine can be injected into the healthy laying hens to develop protective level of antibodies which can be transferred through the eggs of hen. The egg yolk of the immunized hen can be fed to the newly hatched fish try for a period of two weeks at the hatchery and pond level to develop passive immunity. As fishes does not have IgG so the level of predominant IgM and IgY present in the egg yolk and the IgM which is transferred from the brood mother fish to the fry might protect fishes successfully. Biological control measure (using effective fish vaccines injectable or feed based) could be highly effective to reduce or control the infectious bacterial and viral diseases in cultured fin fishes and shrimp in Bangladesh. Following the efficacy of the newly developed injectable form of fish vaccines feed based fish vaccines are ready and going to apply at the farm level soon to increase fish immunity through GILT system.

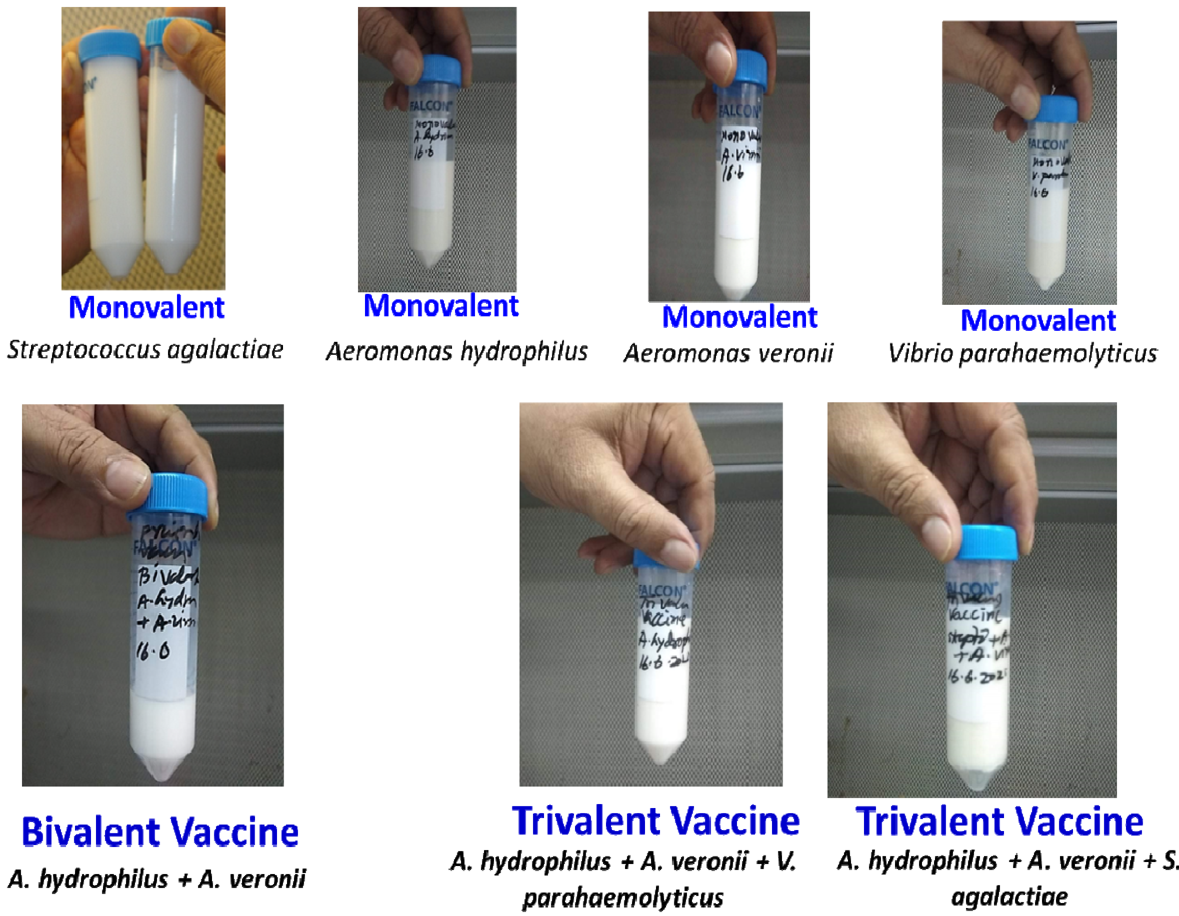


Plate 30. Experimentally prepared inactivated bacterial fish vaccines.

Table 10. Laboratory test results of pathogenicity of the isolated bacteria for healthy Tilapia fishes after experimentally induced infection

Experimental groups	Hosts	No. of fishes used per trial	Bacterial isolate and 0.3 ml/dose of inoculum	Routes inoculated bacteria and PBS		Number and % mortality of fishes (Day 1-day 6 of p.i.)		Number and % of sick/recovered fishes (Day 7-day 14)	
				I/M	Mock control group I/M injection of PBS(0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)
1st trial	Tilapia fish Group-1	10	*A. veronii* (2.3X10^6 CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	4 (80%)	0	1 (20%)	0
1st trial	Tilapia fish Group-2	10	*A. hydrophila* (1.3X10^6 CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Tilapia fish Group-3	10	*S. agalactiae* (0.3 ml/dose of 6.3X10^6 CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Tilapia fish Group-4	10	*V. parahaemolyticus* (8.6X10^6 CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0

Table 11. Laboratory test results of pathogenicity of the isolated bacteriafor healthy V. Koi fishes after experimentally induced infection

Experimental groups	Hosts	No. of fishes used per trial	Bacterial isolate and 0.3 ml/dose of inoculum	Routes inoculated bacteria and PBS		Number and % mortality of fishes (Day 1-day 6 of p.i.)		Number and % of sick/recovered fishes (Day 7-day 14)	
				I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)
1st trial	V. Koi fish Group-5	10	*A. veronii* ($2.3X10^6$ CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	V. Koi fish Group-6	10	*A. hydrophila* ($1.3X10^6$ CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	V. Koi fish Group-7	10	*S. agalactiae* (0.3 ml/dose of $6.3X10^6$ CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	V. Koi fish Group-8	10	*V. parahaemolyticus* ($8.6X10^6$ CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0

Table 12. Laboratory test results of pathogenicity of the isolated bacteriafor healthy Shing fishes after experimentally induced infection

Experimental groups	Hosts	No. of fishes used per trial	0.3 ml/dose of isolated bacterial inoculum	Routes inoculated bacteria and PBS		Number and % mortality of fishes (Day 1-day 6 of p.i.)		Number and % of sick/recovered fishes (Day 7-day 14)	
				I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)
1st trial	Shing fish Group-9	10	*A. veronii* (2.3X10^6 CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Shing fish Group-10	10	*A. hydrophila* (1.3X10^6 CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Shing fish Group-11	10	*S. agalactiae* (0.3 ml/dose of 6.3X10^6 CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Shing fish Group-12	10	*V. parahaemolyticus* (8.6X10^6 CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0

Table 13. Laboratory test results of pathogenicity of the isolated bacteriafor healthy Magur fishes after experimentally induced infection

Experimental groups	Hosts	No. of fishes used per trial	0.3 ml/dose of isolated bacterial inoculum	Routes inoculated bacteria and PBS		Number and % mortality of fishes (Day 1-day 6 of p.i.)		Number and % of sick/recovered fishes (Day 7-day 14)	
				I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)
1st trial	Magur fish Group-13	10	*A. veronii* ($2.3X10^6$ CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Magur fish Group-14	10	*A. hydrophila* ($1.3X10^6$ CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Magur fish Group-15	10	*S. agalactiae* (0.3 ml/dose of $6.3X10^6$ CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	4 (80%)	0	1 (20%)	0
1st trial	Magur fish Group-16	10	*V. parahaemolyticus* ($8.6X10^6$ CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0

Table 14. Laboratory test results of pathogenicity of the isolated bacteriafor healthy Pangas fishes after experimentally induced infection

Experimental groups	Hosts	No. of fishes used per trial	0.3 ml/dose of isolated bacterial inoculum	Routes inoculated bacteria and PBS		Number and % mortality of fishes (Day 1-day 6 of p.i.)		Number and % of sick/recovered fishes (Day 7-day 14)	
				I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)	I/M	Mock control group I/M injection of PBS (0.3ml/ dose)
1st trial	Pangas fish Group-17	10	*A. veronii* ($2.3X10^6$ CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	4 (80%)	0	1 (20%)	0
1st trial	Pangas fish Group-18	10	*A. hydrophila* ($1.3X10^6$ CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Pangas fish Group-19	10	*S. agalactiae* (0.3 ml/dose of $6.3X10^6$ CFU/ml)	5	5	4 (80%)	0	1 (20%)	0
2nd trial		10		5	5	4 (80%)	0	1 (20%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0
1st trial	Pangas fish Group-20	10	*V. parahaemolyticus* ($8.6X10^6$ CFU/ml)	5	5	5 (100%)	0	0 (0%)	0
2nd trial		10		5	5	5 (100%)	0	0 (0%)	0
3rd trial		10		5	5	5 (100%)	0	0 (0%)	0

11.11. Efficacy of the newly developed fish vaccines by immunization and challenge experiment at BFRI, Mymensingh

Results of trials on efficacy of the newly developed fish vaccine types (Monovalent, Bivalent and Tri-valent) by immunization and challenge experiment on different fish group presented in Tables 15-19. Findings of the experiment revealed that the doses 0.3 ml and 0.5 ml resulted similar effectiveness. Therefore, finally the dose of 0.3 ml was selected for field trials.

Table 15. Performance of developed vaccines by immunization and challenge experiment for Tilapia

The experimental fish group in aquarium	Vaccine type	No. of fish	Vaccine dose (ml/fish)	Marking of each fish group	Artificial infection (CFU/ml)	Remarks
Group 1	**Monovalent vaccine** (*S. agalactiae*)	5	0.1	Caudal fin cut	*S. agalactiae* ($6.3X10^6$)	Sign and symptoms of erratic movement observed. Fish mortality not found
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 2	**Monovalent vaccine** *(A. veronii)*	5	0.1	Caudal fin cut	*A. veronii* ($2.3X10^6$)	Same sign and symptoms of skin ulcer found in 5 fish: Mortality 1 fish
		5	0.3	Dorsal fin cut		ulcerative symptoms developed in 2 fish: Recovered
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 3	**Bivalent vaccine** *(A. hydrophila + A. veronii)*	5	0.1	Caudal fin cut	*A. hydrophila* ($1.3X10^6$) *A.veronii* ($2.3X10^6$)	Same sign and symptoms of skin ulcer found in 4 fish: Mortality 1 fish
		5	0.3	Dorsal fin cut		Hemorrhage at Injection site; Recovered
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 4	**Tri-valent vaccine *(A. hydrophila + A. veronii + S. agalactiae)***	5	0.1	Caudal fin cut	*S. agalactiae* ($6.3X10^6$), *A. hydrophila* ($1.3X10^6$) and *A. veronii* $2.3X10^6$	Ulcerative sign and symptoms found in 5 fish: Mortality 2 fish
		5	0.3	Dorsal fin cut		Hemorrhage at Injection site; Recovered
		5	0.5	Pectoral fin cut		Hemorrhage at Injection site; Recovered
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 5	**Tri-valent vaccine *(A. hydrophila + A. veronii + V. parahaemolyticus)***	5	0.1	Caudal fin cut	*A. hydrophila*($1.3X10^6$), *A. veronii*($2.3X10^6$) and *V. parahaemolyticus* ($8.6X10^6$)	Same sign and symptoms at 5 fishes:
		5	0.3	Dorsal fin cut		Hemorrhage at injection site of 1 fish; Recovered
		5	0.5	Pectoral fin cut		No diseases symptom found. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**

Table 16. Performance of developed vaccines by immunization and challenge experiment for V. Koi

The experimental fish group in aquarium	Vaccine type	No. of fish	Vaccine dose (ml/fish)	Marking of each fish group	Artificial ifection (CFU/ml)	Remarks
Group 6	**Monovalent vaccine** (*S. agalactiae*)	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6)	Sign and symptoms of erratic movement observed in 2 fish. Fish mortality not found.
		5	0.3	Dorsal fin cut		Hemorrhage at injection site in one fish; Recovered
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 7	**Monovalent vaccine** *(A. veronii)*	5	0.1	Caudal fin cut	*A.veronii* (2.3X10^6)	Same sign and symptoms in 4 fishes:
		5	0.3	Dorsal fin cut		Hemorrhage at injection site of 2 fish; Recovered
		5	0.5	Pectoral fin cut		No diseases symptom found. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 8	**Bivalent vaccine *(A. hydrophila + A. veronii)***	5	0.1	Caudal fin cut	*A. hydrophila* (1.3X10^6) *A.veronii* (2.3X10^6)	Skin Hemorrhagic symptoms at 4 fishes: Mortality 2 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site of 1 fish; Recovered
		5	0.5	Pectoral fin cut		No diseases symptom found. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 9	**Trivalent vaccine *(A. hydrophila + A. veronii + S. agalactiae)***	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A. hydrophila* (1.3X10^6) and *A. veronii* 2.3X10^6	Skin Hemorrhagic symptoms at 1fishes: Mortality 1 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 10	**Tri-valent vaccine *(A. hydrophila + A. veronii + V. parahaemolyticus)***	5	0.1	Caudal fin cut	*A. hydrophila* (1.3X10^6), *A. veronii* (2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Same sign and symptoms at 5 fishes: Mortality 2 fishes
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered. No mortality
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**

Table 17. Performance of developed vaccines by immunization and challenge experiment for Shing

The experimental fish group in aquarium	Vaccine type	No. of fish	Vaccine dose (ml/fish)	Marking of each fish group	Artificial infection (CFU/ml)	Remarks
Group 11	**Monovalent vaccine** (*S. agalactiae*)	5	0.1	Caudal fin cut	*S. agalactiae* ($6.3X10^6$)	Sign and symptoms of haemorrhage on the body of the fishes observed in 2 fishes. One fish died.
		5	0.3	Dorsal fin cut		Not found any disease symptoms in fish. No mortality
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 12	**Monovalent vaccine** *(A. veronii)*	5	0.1	Caudal fin cut	*A.veronii* ($2.3X10^6$)	Ulcerative lesion developed in 4 fishes:
		5	0.3	Dorsal fin cut		Ulcerative lesion developed at Injection site of 2 fish; Recovered
		5	0.5	Pectoral fin cut		Ulcerative lesion developed at Injection site of 1 fish; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 13	**Bivalent vaccine** ***(A. hydrophila + A. veronii)***	5	0.1	Caudal fin cut	*A. hydrophila* ($1.3X10^6$) and *A. veronii* ($2.3X10^6$)	Same sign and symptoms developed in 4 fishes: Mortality 2 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered. No mortality
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 14	**Tri-valent vaccine** *(A. hydrophila + A. veronii + S. agalactiae)*	5	0.1	Caudal fin cut	*S. agalactiae* ($6.3X10^6$), *A. hydrophila* ($1.3X10^6$) and A.*veronii* ($2.3X10^6$)	Ulcerative lesion and symptoms found in 5 fish: Mortality 3 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered Mortality 1 Fish.
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 15	**Tri-valent vaccine** *(A. hydrophila + A. veronii + V. parahaemolyticus)*	5	0.1	Caudal fin cut	*A. hydrophila* ($1.3X10^6$), *A. veronii*, $2.3X10^6$) and *V. parahaemolyticus* ($8.6X10^6$)	Ulcerative lesion and symptoms found in 4 fishes: Mortality 1 fishes
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered. No mortality
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**

Table 18. Performance of developed vaccines by immunization and challenge experiment for Magur

The experimental fish group in aquarium	Vaccine type	No. of fish	Vaccine dose (ml/fish)	Marking of each fish group	Artificial infection (CFU/ml)	Remarks
Group 16	**Monovalent vaccine** (*S. agalactiae*)	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6)	Sign and symptoms of haemorrhage on the body of the fishes observed in 2 fishes. Recovered
		5	0.3	Dorsal fin cut		Not found any disease symptoms in fish. No mortality
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 17	**Monovalent vaccine** (*A. veronii*)	5	0.1	Caudal fin cut	*A. veronii* (2.3X10^6)	Skin ulcerative symptoms developed in 4 fish: Mortality 1 fish
		5	0.3	Dorsal fin cut		Ulcerative symptoms developed in 2 fish: Recovered
		5	0.5	Pectoral fin cut		No ulcerative symptoms developed in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 18	**Bivalent vaccine** (*A. hydrophila* + *A. veronii*)	5	0.1	Caudal fin cut	*A. hydrophila* (1.3X10^6) and *A. veronii* (2.3X10^6)	Same sign and symptoms at 2 fishes: Mortality 1 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 19	**Tri-valent vaccine** (*A. hydrophila* + *A. veronii* + *S. agalactiae*)	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A.hydrophila* (1.3X10^6) and *A. veronii* (2.3X10^6)	Same sign and symptoms at 5 fish: Mortality 4 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 20	**Tri-valent vaccine** (*A. hydrophila* + *A. veronii* + *V. parahaemolyticus*)	5	0.1	Caudal fin cut	*A. hydrophila* (1.3X10^6), *A. veronii* (2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Same sign and symptoms at 5 fishes: Mortality 1 fishes
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**

Table 19. Performance of developed vaccines by immunization and challenge experiment for Pangas

The experimental fish group in aquarium	Vaccine type	No. of fish	Vaccine dose (ml/fish)	Marking of each fish group	Artificial infection (CFU/ml)	Remarks
Group 21	**Monovalent vaccine** (*S. agalactiae*)	5	0.1	Caudal fin cut	*S. agalactiae* ($6.3X10^6$)	Hemorrhage on the body of the fishes observed in 1 fish. Recovered
		5	0.3	Dorsal fin cut		Not found any disease symptoms in fish. No mortality
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 22	**Monovalent vaccine** *(A. veronii)*	5	0.1	Caudal fin cut	*A. veronii* ($2.3X10^6$)	Skin ulcerative symptoms developed in 4 fish: Mortality 1 fish
		5	0.3	Dorsal fin cut		Ulcerative symptoms developed in 2 fish: Recovered
		5	0.5	Pectoral fin cut		No ulcerative symptoms developed in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 23	**Bivalent vaccine** *(A. hydrophila + A. veronii)*	5	0.1	Caudal fin cut	*A. hydrophila* ($1.3X10^6$) and *A. veronii* ($2.3X10^6$)	Same sign and symptoms of skin ulcer found in 5 fish: Mortality 1 fish
		5	0.3	Dorsal fin cut		Ulcerative symptoms developed in 2 fish: Recovered
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 24	**Tri-valent vaccine** *(A. hydrophila + A. veronii + S. agalactiae)*	5	0.1	Caudal fin cut	*S. agalactiae* ($6.3X10^6$), *A. hydrophila* ($1.3X10^6$) and *A. veronii* $2.3X10^6$	Same sign and symptoms of skin lesion developed in 5 fish: Mortality 3 fish
		5	0.3	Dorsal fin cut		Lesion developed in 1 fish; No mortality found
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
Group 25	**Tri-valent vaccine** (*A. hydrophila + A. veronii + V. parahaemolyticus)*	5	0.1	Caudal fin cut	*A. hydrophila* $1.3X10^6$, *A. veronii*, $2.3X10^6$ *V. parahaemolyticus* $8.6X10^6$	Same sign and symptoms of skin lesion developed in 4 fish: Mortality 1 fish
		5	0.3	Dorsal fin cut		Ulcerative symptoms developed in 1 fish: Recovered
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No mortality found
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**

11.12. Results of antibody titre of vaccines

A. Tilapia fishes

Fig 2 showing serum antibody (IgM) response of Tilapia fishes following oral, IP and IM vaccination with locally isolated *S. agalactiae* (GBS) killed vaccine. Findings revealed that among the three routes with two boosters of vaccination at week 2 and 6 prolonged the high antibody levels in the IM route for up to 11 weeks compared to oral and IP routes in Tilapia fish (*Oreochromis niloticus*).

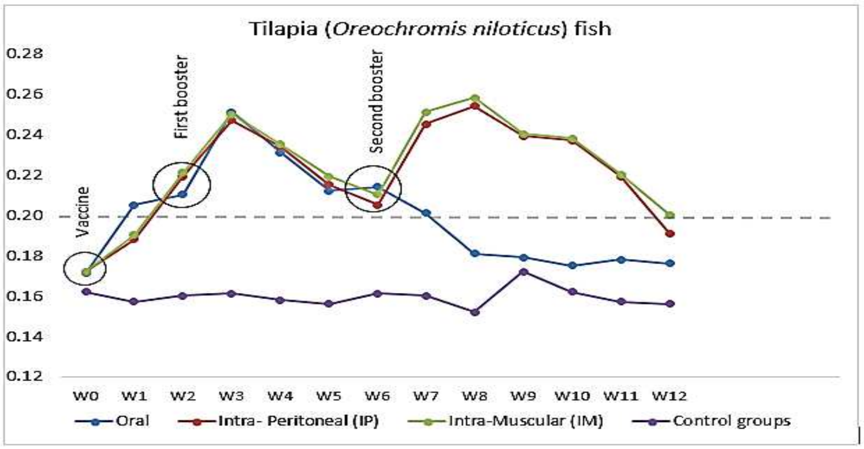


Fig. 2. Serum antibody (IgM) response of Tilapia fish.

B. Vietnamese Koi fishes

Fig 3 Showing serum antibody (IgM) response of Vietnamese Koi fishes following oral, IP and IM vaccination with locally isolated *S. agalactiae* (GBS) killed vaccine. Findings revealed that among the three routes with two boosters of vaccination at week 2 and 6 prolonged the high antibody levels in the IM route for up to 12 weeks compared to oral and IP routes in Vietnamese Koi fish (*Anabas testudineus*) fish.

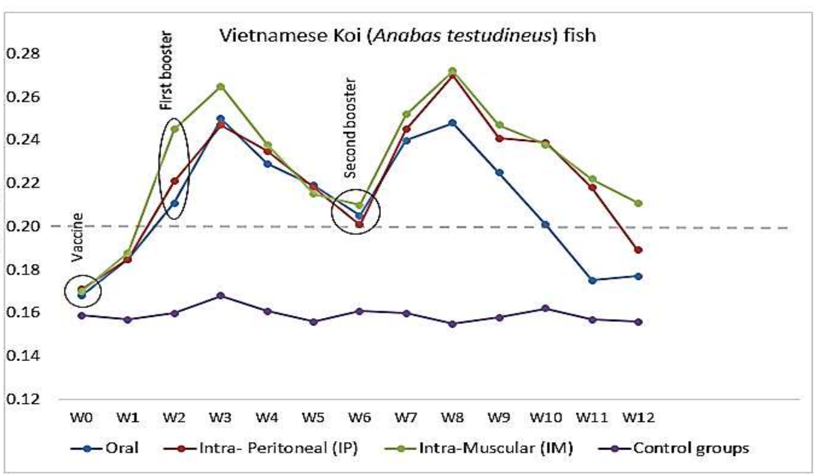


Fig. 3. Serum antibody (IgM) response of Vietnamese Koi fish.

C. Shing fish

Fig. 4 Showing serum antibody (IgM) response of Shing fish following oral, IP and IM vaccination with locally isolated *S. agalactiae* (GBS) killed vaccine. Findings revealed that among the three routes with two boosters of vaccination at week 2 and 6 prolonged the high antibody levels in the IM route for up to 12 weeks compared to oral and IP routes in Shing fish (*Heteropneustes fossilis*).

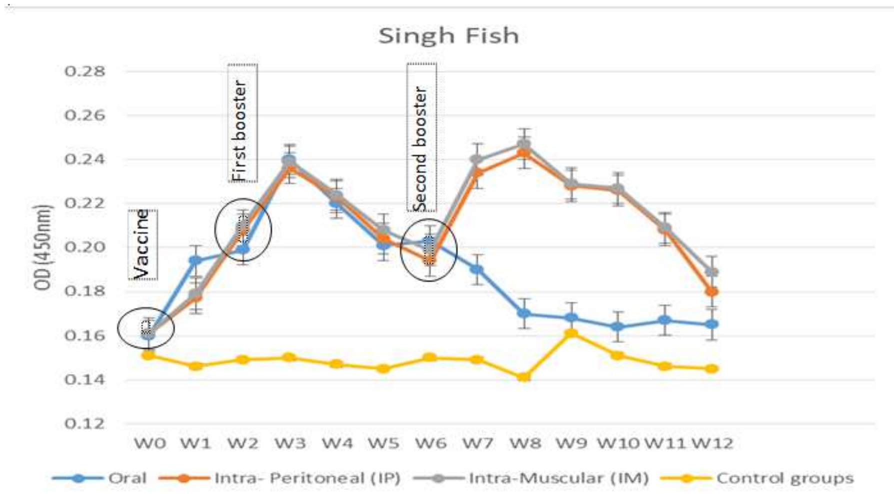


Fig. 4. Serum antibody (IgM) response of Shing fish.

D. Magur fish

Fig 5 Showing serum antibody (IgM) response of Magur fish following oral, IP and IM vaccination with locally isolated *S. agalactiae* (GBS) killed vaccine. Findings revealed that among the three routes with two boosters of vaccination at week 2 and 6 prolonged the high antibody levels in the IM route for up to 12 weeks compared to oral and IP routes in Magur fish (*Clariasbatrachus*).

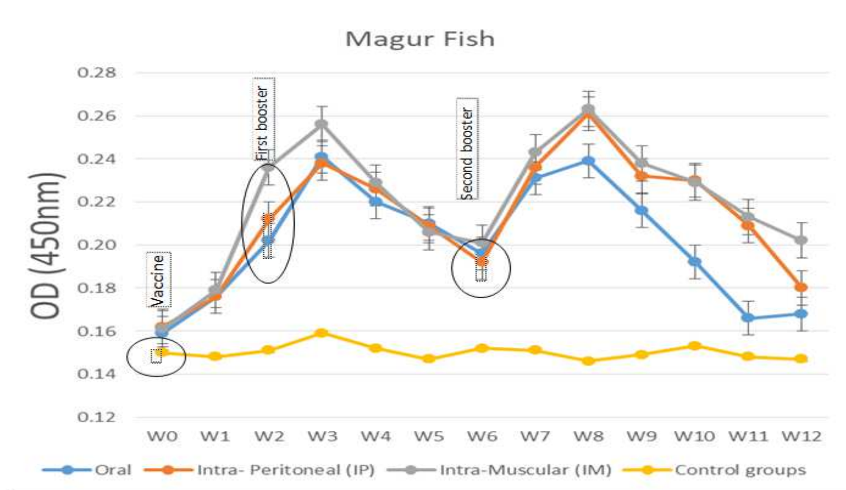


Fig. 5. Serum antibody (IgM) response of Magur fish.

E. Pangas fish

Fig. 6. Showing serum antibody (IgM) response of Pangas fish following oral, IP and IM vaccination with locally isolated *S. agalactiae* (GBS) killed vaccine. Findings revealed that among the three routes with two boosters of vaccination at week 2 and 6 prolonged the high antibody levels in the IM route for up to 12 weeks compared to oral and IP routes in Pangas fish (*Pangasius hypophthalmus*).

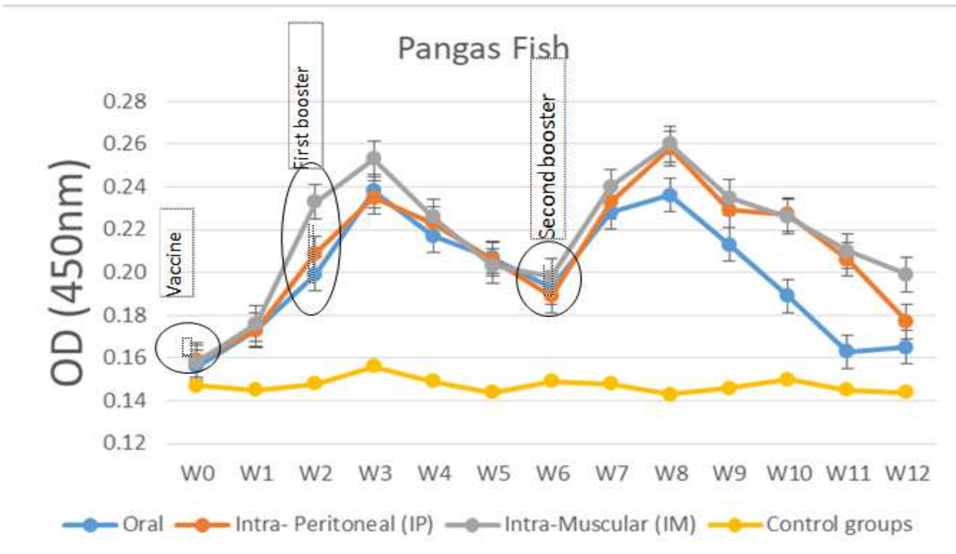


Fig. 6. Serum antibody (IgM) response of Pangas fish.

The serum IgM antibody levels of all groups of fishes before vaccination showed insignificant (p>0.05) differences. Following vaccination with oral, IP and IM vaccinated groups showed significantly (p<0.05) higher IgM levels than the control group. The oral, IP and IM routes of vaccination at week 2, the increasing pattern continued for all the three vaccinated groups and the antibody level reached the peak at week 3. The antibody level of fishes after first vaccination started to decrease insignificantly (p>0.05) within4-5th weeks in the fishes of all groups but the fishes vaccinated with the oral route in Tilapia, V. Koi, Shing, Magur and Pangas fishes decreased significantly (p<0.05) which was non-protective level and the cut-off value was (p>0.05) at week 6, compare to that of IP and IM routes of vaccination (Figs. 2-6).

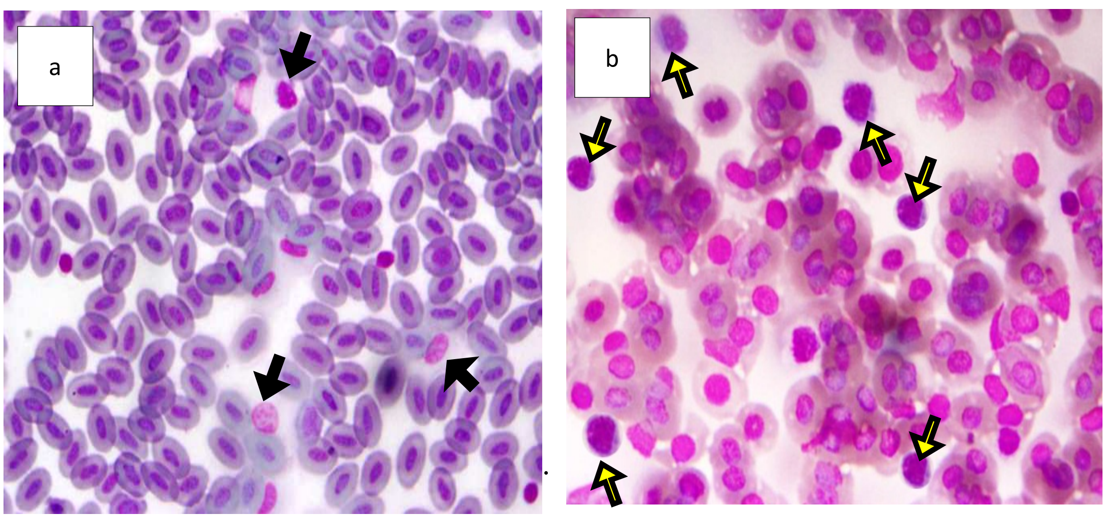
However, following the second booster in week 6 of age, the antibody level increased significantly (p<0.05) in IM and IP routes of vaccination in all species of fishes again the level reached the peak at week 8, serum antibody level of all the vaccinated groups of fishes started to decline at 11weeks of (Figs. 2-6). The IM and IP routes of vaccinated group of Tilapia, V. Koi, Shing, Magur and Pangas fishes showed rapid rate of declination of IgM levels (p> 0.05) by week 9th, week10th and week 11th. The cut-off value of serum antibody level remained lower until the end of the experimental period 12 weeks. In the control groups, the serum antibody levels was found significantly lower (p<0.05) than the protective cut-off value for all species of fin fishes used in this study (Figs. 2-6).

The result of the present study has similarity with the findings of Ismail *et al*. (2016). They also found that the serum antibody level of the fishes vaccinated through IM route did not go under the level of protection when the fishes were vaccinated through primary vaccination followed by two booster doses. Findings of this study highly agree with the findings of Pasaribu *et al.,* (2018). They found that immunizations of brood stock through IM route using a monovalent whole cell vaccine of *S. agalactiae* provide effective protection when challenged with *S. agalactiae* bacteria.

11.13 Cconcentration of lymphocytes in blood of vaccinated and non-vaccinated fishes

A. Tilapia fish (*Oreochromis niloticus*)

Plate 31 showing blood film of Tilapia (*Oreochromis niloticus*) fish 100 x magnifications. In figure "a" non-vaccinated control fish showing infiltration of lymphocytes along with nucleated red blood cells (black arrow). Whereas figure "b" (Vaccinated fish) shows more lymphocytic infiltration (yellow arrow) along with nucleated red blood cells.

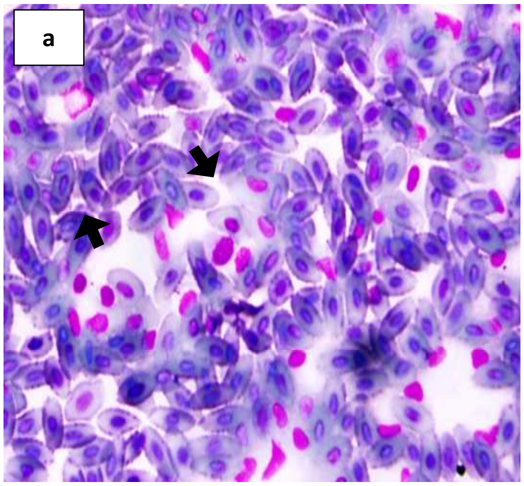


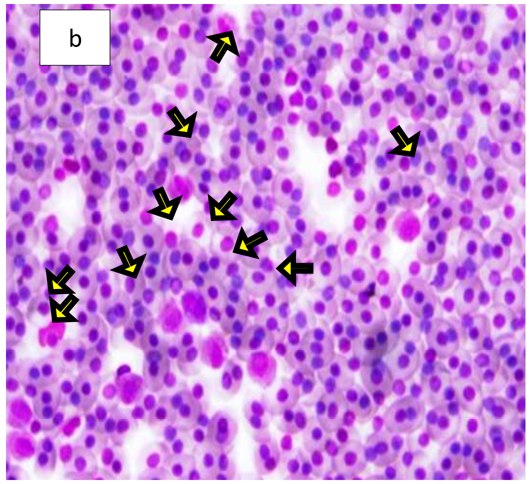
Control fish **Vaccinated fish**

Plate 31. Showing blood film of Tilapia fish with 100 x magnifications.

B. Vietnamese koi fish (*Anabas testudineus*)

Plate 32 showing blood film of Vietnamese koi (*Anabas testudineus*) fish 40 x magnifications. In figure "**a**" non-vaccinated control fish showing infiltration of lymphocytes along with nucleated red blood cells (black arrow). Whereas figure "**b**" (Vaccinated fish) shows more lymphocytic infiltration (yellow arrow) along with nucleated red blood cells.



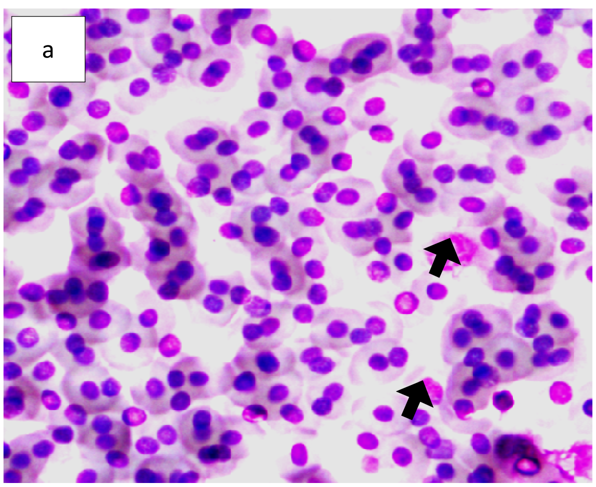


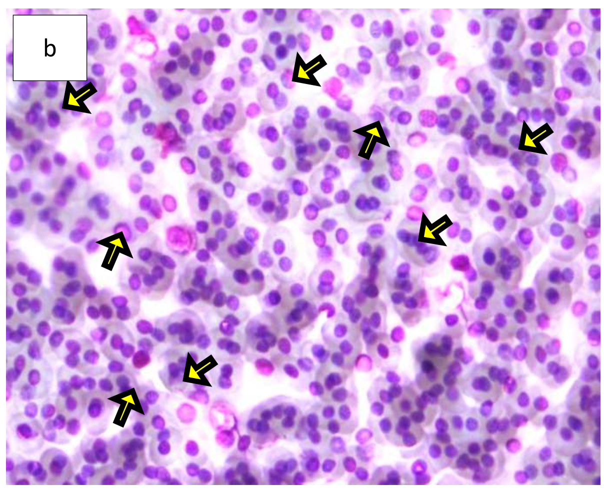
Control fish | Vaccinated fish

Plate 32. Showing blood film of Vietnamese koi fish 40 x magnifications.

C. Shing fish (*Heteropneustes fossilis*)

Plate 33 showing blood film of Shing (*Heteropneustes fossilis*) fish 40 x magnifications. In figure "**a**" non-vaccinated control fish showing infiltration of lymphocytes along with nucleated red blood cells (Black arrow). Whereas figure "**b**" (Vaccinated fish) shows more lymphocytic infiltration (yellow arrow) along with nucleated red blood cells.



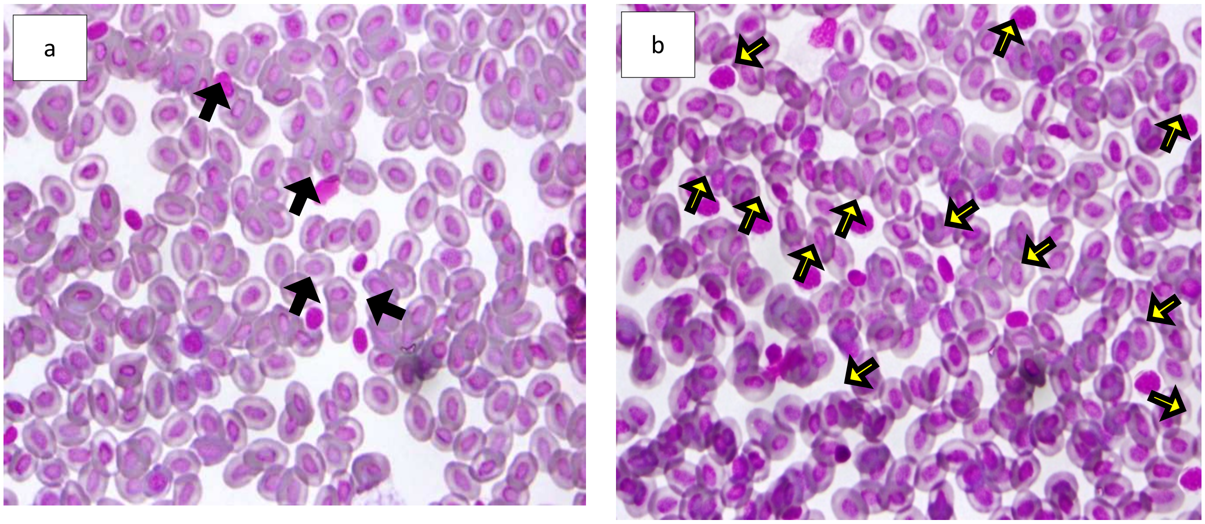


Control fish | Vaccinated fish

Plate 33. Showing blood film of Shing fish with 40 x magnifications.

D. Magur (*Clarias batrachus*)

Plate 34 showing blood film of Magur (*Clarias batrachus*) fish 40 x magnifications. In figure "**a**" non-vaccinated control fish showing infiltration of lymphocytes along with nucleated red blood cells (black arrow). Whereas figure "**b**" (Vaccinated fish) shows more lymphocytic infiltration (yellow arrow) along with nucleated red blood cells.

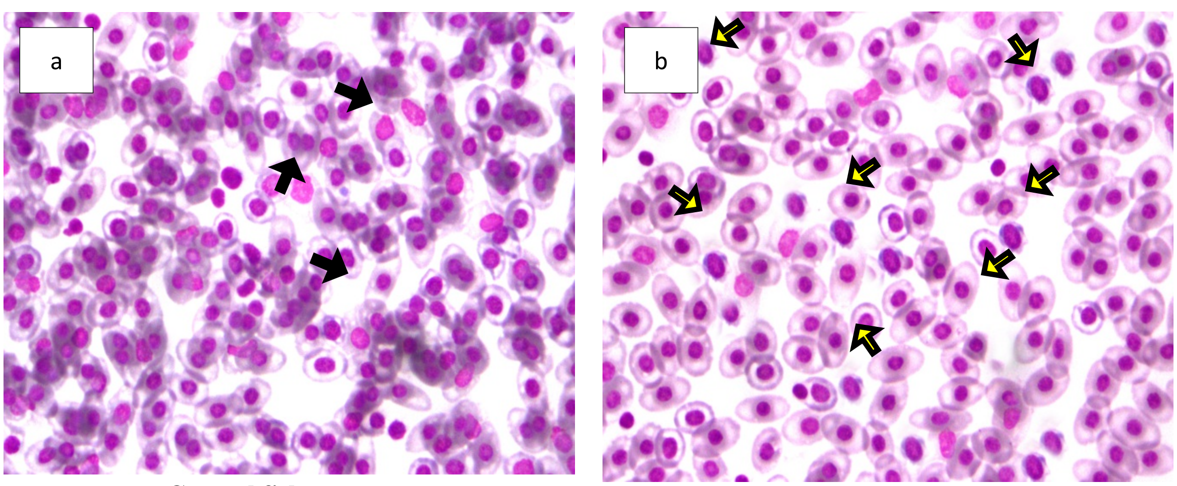


Control fish **Vaccinated fish**

Plate 34. Showing blood film of Magur fish with 40 x magnifications.

E. Pangas (*Pangasius hypophthalmus*)

Plate 35 showing blood film of Pangas (*Pangasius hypophthalmus*) fish 40 x magnifications. In figure "**a**" non-vaccinated control fish showing infiltration of lymphocytes along with nucleated red blood cells (black arrow). Whereas figure "**b**" (Vaccinated fish) shows more lymphocytic infiltration (yellow arrow) along with nucleated red blood cells.



Control fish **Vaccinated fish**

Plate 35. Showing blood film of Pangas fish with 40 x magnifications.

The levels of leukocytes, lymphocytes, monocytes, and granulocytes were significantly higher ($p < 0.05$) in the fishes vaccinated through IM route of vaccination than that of IP and oral routes in Tilapia, V. Koi, Shing, Magur and Pangas fishes after vaccination of primary and two booster doses (Plates 31-35). The leukocyte count is one of the most significant haematological indicators for the fish's non-specific and specific immune systems (Misra *et al*., 2006). Results of the present study agreed with the findings of Monir *et al*. (2020). They also found that the number of leucocytes, lymphocytes, monocytes and granulocytes were significantly increased ($p<0.05$) in the vaccinated groups at 21 days of post-vaccination compared to that of unvaccinated control group.

11.14. Results of challenge test of fishes larvae after egg yolk feeding

The RPS was determined at day 7, day 14 and day 21 in the vaccinated groups of fishes. There was significantly difference ($p<0.05$) was noticed in the fish larvae immunized with egg yolk feeding than the broodstock without immunization during challenged with the *S. agalactiae*. Larvae of 21 days feeding found the highest RPS in both Tilapia (81.75%) and V. Koi (86.64%) fishes compared to 7 and 14 days after egg yolk feeding. In both the Tilapia and V. Koi fish, the RPS was found to be considerably greater as the larval age increased.

Lu *et al.* (2009) stated that the RPS of passively immunized larvae with egg yolk feeding found relatively higher than control. This result is also inversely related to the study when immunized larvae were produced from immunized brood stock. Brood stock immunization can probably improve the quality of offspring it produces. Cao *et al.,* (2017) reported that the larvae of 20 days ofage already exist differentiation in the lymphoid organ thymus. Firdausi and Nuryati (2017) found that the re-immunization of Tilapia at 20 days after hatching increased larval protection. The re-immunization treatment in fish has also been shown to improve fish survivability (Chettri *et al.,* 2015 and Pretto-Giordano *et al.,* 2010).

11.15. Efficacy of the newly developed tetra-valent fish vaccine by immunization and challenge experiment at Lab and field trial

Results of efficacy of the newly developed tetra-valent fish vaccine by immunization and challenge experiment at Lab and field trial are presented in Table 21. Findings revealed that the tetravalent fish vaccine was found highly effective at the doses of 0.3 ml and 0.5 ml. So, from the two doses we choose the 0.3 ml/fish dose of tetravalent vaccine in the field trials.

Table 20. Results of efficacy of the newly developed tetra-valent fish vaccine by immunization and challenge experiment at Lab and field trial

For Tilapia fish						
The experimental fish group in aquarium	**Vaccine type**	**No. of fish**	**Vaccine dose (ml/fish)**	**Marking of each group of vaccinated and non-vaccinated fishes**	**Challenge dose for each species of bacteria (CFU/ml)**	**Remarks**
Group 5	**Tetra-valent vaccine** (*S. agalactiae A. hydrophila* + *A. veronii* + *V. parahaemolyticus)*	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A. hydrophila* (1.3X10^6), *A. veronii* (2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Similar sign and symptoms found in 5 fishes.
		5	0.3	Dorsal fin cut		Hemorrhage was observed in 1 fish at the site of injection, followed by recovery.
		5	0.5	Pectoral fin cut		No diseases symptom found. No fish mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
For V. Koi fish						
Group 10	**Tetra-valent vaccine** (*S. agalactiae A. hydrophila* + *A. veronii* + *V. parahaemolyticus)*	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A. hydrophila* (1.3X10^6), *A. veronii*(2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Similar signs and symptoms were observed in 5 fishes. Two fish died.
		5	0.3	Dorsal fin cut		Hemorrhage was observed in 1 fish at the site of injection, followed by recovery. No fish mortality found.
		5	0.5	Pectoral fin cut		Hemorrhage was observed in 1 fish at the site of injection, followed by recovery. No fish mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
For Shing fish						
Group 15	**Tetra-valent vaccine** (*S. agalactiae A. hydrophila* + *A. veronii* + *V. parahaemolyticus)*	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A. hydrophila* (1.3X10^6), *A. veronii*(2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Ulcerative lesions and symptoms found in 4 fishes: Mortality 1 fishes
		5	0.3	Dorsal fin cut		No fish mortality
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered. No mortality
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
For Magur fish						
Group 19	**Tetra-valent vaccine** (*S. agalactiae A. hydrophila* + *A. veronii* + *V. parahaemolyticus)*	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A. hydrophila* (1.3X10^6), *A. veronii*(2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Ulcerative lesions with similar sign and symptoms at 5 fish: Mortality 4 fish
		5	0.3	Dorsal fin cut		Hemorrhage at injection site; Recovered
		5	0.5	Pectoral fin cut		Hemorrhage at injection site; Recovered
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**
For Pangas fish						
Group 24	**Tetra-valent vaccine** (*S. agalactiae A. hydrophila* + *A. veronii* + *V. parahaemolyticus)*	5	0.1	Caudal fin cut	*S. agalactiae* (6.3X10^6), *A. hydrophila* (1.3X10^6), *A. veronii*(2.3X10^6) and *V. parahaemolyticus* (8.6X10^6)	Five fish developed the same signs and symptoms of a skin lesion. Mortality 3 fish
		5	0.3	Dorsal fin cut		lesion developed in 1 fish; No fish mortality found
		5	0.5	Pectoral fin cut		Not found any disease symptoms in fish: No fish mortality observed
Control group	**Non vaccinated**	5	**PBS**	N/A		**100% mortality**

11.16. Results of antibody titre of fish serum vaccinated with tetravalent killed vaccines

The serum antibody (IgM) response of fishes vaccinated with tetravalent killed vaccine following IM, IP and oral routes of vaccination with locally isolated *S. agalactiae* (GBS), *A. hydrophila, A. veronii* and *V. parahaemolyticus* was measured by ELISA. Among the three routes with two boosters vaccination at week 2 and 6 prolonged the antibody levels in the IM route for up to 11 weeks compared to those of oral and IP routes in Tilapia, V. Koi, Shing, Magur and Pangas fishes. The serum IgM antibody levels of all groups of fishes before vaccination showed insignificant (p>0.05) differences. Following vaccination with IM, IP and oral vaccinated groups showed significantly (p<0.05) higher IgM levels than the control group. The oral, IP and IM routes of vaccination at week 2, the increasing pattern continued for all the three vaccinated groups and the antibody level reached the peak at week 3. The antibody level of fishes after first vaccination started to decrease insignificantly (p>0.05) within 4-5th weeks in the fishes of all groups but the fishes vaccinated with the oral route in Tilapia, V. Koi, Shing, Magur and Pangas fishes decreased significantly (p<0.05) which was non-protective level and the cut-off value was (p>0.05) at week 6, compere to that of IP and IM routes of vaccination. Whereas, in the following the second booster in week 6 of age, the antibody level increased significantly (p<0.05) in IM and IP routes of vaccination in all five species of fishes and the level reached peak at week 8, serum antibody level of all the vaccinated groups of fishes started to decline at 11 weeks of age. The IM and IP routes of vaccinated group of Tilapia, V. Koi, Shing, Magur and Pangas fishes showed rapid rate of declination of IgM levels (p>0.05) by week 9th, week10th and week 11th. The result of the present study has similarity with the findings of Ismail *et al.* (2016). They also found that the serum antibody level of the fishes vaccinated through primary vaccination followed by two booster doses through IM route, the level of protection was found higher than that of single vaccination.

11.17. Results of lymphocytes count of blood of brood vaccinated (tetravalent) and non-vaccinated fishes

The lymphocytes count of the blood of brood Tilapia, V. Koi, Shing, Magur and Pangas fishes vaccinated with tetravalent vaccine and non-vaccinated control fishes were observed at 100X magnifications stained by modified wright stain. The brood fishes of all the vaccinated groups lymphocytic infiltration in their blood were higher along with nucleated red blood cells compared to that of non-vaccinated control fishes.The levels of leukocytes, lymphocytes, monocytes, and granulocytes were significantly higher (p< 0.05) in the fishes vaccinated through IM route of vaccination than that of IP and oral routes in Tilapia, V. Koi, Shing, Magur and Pangas fishes after vaccination of primary and two booster doses. Results of the present study agreed with the findings of Monir *et al.* (2020). They also found that the number of leucocytes, lymphocytes, monocytes and granulocytes were significantly increased (p<0.05) in the blood of vaccinated groups of fishes at 21 days of post-vaccination compared to that of unvaccinated control group.

11.18. Results of challenge test of fishes larvae derived from vaccinated Tilapia, V. Koi, Shing, Magur and Pangas fishes after feeding of egg yolk of the immuized hens

The RPS was determined at day 7, day 14 and day 21 in the larvae of tetravalent vaccinated groups of fishes. A significant difference (p<0.05) was noticed in the fish larvae derived from immunized group and fed with egg yolk than that of the brood stock without immunization during challenged with the four bacteria (*S. agalactiae* (GBS), *A. hydrophila, A. veronii* and *V. parahaemolyticus*). Larvae of 21 days feeding found highest RPS in Tilapia (78.95%), V. Koi (83.75%), Shing (88.21%), Magur (85.67%) and Pangas (90.44%) fishes compared to the larvae of 7 and 14 days after feeding of egg yolk. Following feeding with immunized hen's egg yolk, the RPS of larvae from all fish groups vaccinated with tetravalent fish vaccine was observed to be significantly higher as the larval age increased until 21 days of observation. Lu *et al.* (2009) stated that the RPS of passively immunized larvae with egg yolk feeding found relatively higher than the control. This result is also inversely related to the study when immunized larvae were produced from immunized brood fishes. Brood stock immunization can probably improve the quality of offspring it produces. Firdausi and Nuryati (2017) found that the re-immunization of Tilapia at 20 days after hatching increased larval protection. The re-immunization treatment in fish has also been shown to improve fish survivability (Chettri *et al.,* 2015 and Pretto-Giordano *et al.,* 2010).

11.19. Statistical analysis on the efficacy of experimentally developed fish vaccines

All data from three replicates were given as means standard error (M±SE). The RPS was statistically analysed by comparing the immunization group's therapy with a challenge test using the same pathogenic bacteria and stage. One-way ANOVA with Duncan's Multiple Range Test (DMRT) was used to assess differences in haemato-immunological parameters and RPS between the unvaccinated and vaccinated groups. SPSS Software was utilized for the statistical analysis (version 24.0 for Windows, SPSS Inc., Chicago, IL, USA).

12. Research highlights

Title of sub project: ***Investigation and characterization of viral and bacterial diseases in selected fin fishes and Shrimp in Bangladesh and development of their vaccines and validation***

Background

Aquaculture in Bangladesh is growing rapidly with respect to both quantity and variety of fish species. From 2000 and 2016, aquaculture production increased from 712,640 and 2,060,408 metric tons which is much larger quantity than wild capture production (1.023 million tons) in 2015. Pond culture represents the mainstay of aquaculture in Bangladesh, accounting for 85.8% of the total recorded production and 57.7% of the area under culture. The current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. However, Tilapia (*Oreochromis niloticus*), Koi (*Anabas testudineus*), Shing (*Heteropneustesfossilis*), Magur (*Clariasbatrachus*) Pangas (*Pangasius hypophthalmus*) and

Shrimp (*Penaeus monodon*) are cultured in ponds mostly on commercial basis by entrepreneurial farmers in Bangladesh.

But in recent years, most of the farmers reported that they are not getting enough profit from freshwater fin fishes and shrimp culture. This is mainly due to several viral, bacterial, parasitic and fungal diseases of fish that are affecting total growth period (fry to adult) and their productivity in culture system. Most of those viral and bacterial diseases causative agents not yet detected or characterized and their control/preventive measures (through vaccine) are not available yet in Bangladesh.

Objectives

- To investigate the various environmental factors influencing the occurrence of infectious diseases, determine the pathogenicity of the infectious agents
- To isolate, identify and characterize the isolated emerging and existing viruses and bacteria responsible for mass mortality of fin fishes and Shrimp,
- Development of effective fish vaccines and to evaluate efficacy of the experimentally developed vaccines in the specific hosts with the isolated viruses and bacteria.

Methodology

The water temperature, pH, dissolved oxygen, and quantities of unionized ammonia of the water of cultural ponds were considered as effective environmental factors. In this experiment the HACH Kit FF3 was used for measuring the mentioned water quality parameters. Selective bacteriological broth and agar were used for the isolation and identification of the specific bacterial pathogens. Colony morphology on solid agar media andmorphological characteristic with Gram's staining of the bacteria were observed.Different Biochemical tests were conducted for the identification of the isolated bacteria. Catalase, Oxidase, Indole test, Voges–Proskauer (VP) test, Methyl Red (MR) Test, Arginine Dihydrolase (ADH) test, Oxidative-Fermentative (O/F) test, Glucose, Mannitol and Lactose test for *Streptococcus* spp. NO_3 to NO_2, D-Glucose (gas), Indole test, Lysine decarboxylase, 0% and 6% NaCl tolerance, Sucrose, D-Manitol, L-Arabinose, Salicin and Manosetest for *Aeromonas* spp. whereas Oxidase, D–Glucose (gas), Lactose, Sucrose, 0% and 6% NaCl tolerance, Sucrose, Inositol, Lysine decarboxylase, Arginine dihydrolase and Ornithine decarboxylase test for *Vibrio* spp. were conducted for the Biochemical tests for the isolated and identification of the bacteria. The genomic DNA was extracted by the boiling method (Rawool*et al.*, 2007). A single colony of each bacterium was placed into 100 µl of distilled water in an Eppendorf tube, mixed well and then boiled for 10 minutes. After boiling, the tube was immediately placed into the crusted ice for cold shock for 5 minutes followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected and used as template DNA for every species of bacteria. Biological (infecting natural host system) and molecular methods (PCR) were used to detect and characterize the isolated bacteria. Pathogenicity of the isolated bacteria was tested through induced experimental infection into specific species of fishes at BFRI laboratory. Later on inactivated whole cell bacterial vaccines (mono-, bi- and tri-valent) were developed by using the field isolates of bacteria with Montanide adjuvant.Different species of fishes immunized through IM, IP and oral routes were subjected to challenge experiment.

Key findings

- Physico-chemicat water parameters like, water temperature (28^0C -32^0C), pH (7.5-8.5), dissolved oxygen (5.0 mg/L - 10.0 mg/L) and quantities of unionized ammonia (0.00 mg/L - 0.02 mg/L)of the culture ponds were ranged near to optimum levels.
- Isolation, identification and characterization of *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus* bacteria were done from Tilapia, V. Koi, Shing, Magur, Pangas and Shrimp. The bacteria were identified ashighly pathogenic to different species of fishes.
- Four Mono-valent (*S. agalactiae*, *A. hydrophila*, *A. veronii*and *V. parahaemolyticus*), one Bi-valent (*A. hydrophila* + *A. veronii*) and two Tri-valent (*A. hydrophila* + *A. veronii* + *S. agalactiae* and *A. hydrophila* + *A. veronii* + *V. parahaemolyticus*) vaccines were developed
- Efficacy of thenewly developed bacterial mono-valent, bi-valent and tri-valent vaccines and their validation at laboratory and field levelwere completed.The result was found highly satisfactory in terms of morbidity and mortality of the stated fin fishes under the study.

Key words : Water quality parameter; Mono-valent, Bi-valent &Tri-valent; Pathogenicity; Vaccine validation

B. Implementation Status

1. Procurement (Component wise)

Component 1 (BFRI)

Description of equipment and capital items	PP Target		Achievement		Remarks
	Physical (No.)	Financial (Tk.)	Physical (No.)	Financial (Tk.)	
Office Furniture	1	82,500	1 no.	82,500	Target achieves 100% as per plan
Office Equipment	1	150,000	1 no	149,600	
Lab Equipment	1	840,000	1 no	740,000	
Feed	3	1050,000	1 no.	120,200	
Fingerlings	1	500,000	1 no.	432,500	

Component 2 (BAU)

Description of equipment and capital items	PP Target		Achievement		Remarks
	Physical (No.)	Financial (Tk.)	Physical (No.)	Financial (Tk.)	
(a) Office equipment					Target achieves 100% as per plan
Desktop Computer	1 no.	60000	1 no.	59700	
Scanner	1 no.	10000	1 no.	9800	
Printer	1 no.	20000	1 no.	19000	
Laptop	1 no)	60000	1 no)	60000	
Total		**150000**		**148500**	
(b) Lab equipment					
Ultra-low temperature	1 no.	1200000	1 no.	1198000	

freeze					
Thermal Cycler	1 no)	750000	1 no)	745000	
Autoclave machine	1 no	550000	1 no	548000	
Total		**2500000**		**2491000**	
(c) Furniture					
Executive Table	1 no.	20200	1 no.	20000	
Executive chair	1 no.	10000	1 no.	10000	
Visitor chair	5 no.	22500	5 no.	22500	
File cabinet	1 no.	20000	1 no.	20000	
Computer table	1 no.	5500	1 no.	5500	
Computer chair	1 no.	4300	1 no.	4300	
Total		**82500**		**82,300**	
Small transport					
Motorcycle	1 no.	150000	1 No.	149700	
	Total	**150000**		**149700**	

2. Establishment/renovation facilities: *Not applicable*

3. Training/study tour/seminar/*workshop*/conference organized by the sub project

Description	Number of participants			Duration (Days)	Remarks
	Male	Female	Total		
Inception workshop (1 no)	56	7	63	1 day	All workshops held at the Conference room of BARC as per schedule of activity of the Coordination component
Half yearly Research Prog. Review workshop (2 no.)	65+ 62	9+8	144	1+1 = 2 days	
Annual Research Prog. Review workshop (2 no.)	60+63	7+8	138	1+2 =3 days	
Project Completion Report Review workshop (1 no)	45	6	52	1 day	
Training					
BFRI (Comp-1)-01	12	08	20	01 Day	The role of vaccines in fish farming and disease prevention (BFRI, Mymensingh)

C. Financial and Physical Progress (Combined and component wise)

Combined Fig in Tk.

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	3808417	3615939	3347674	268265	87.90	Unspent money refunded to the PIU, BARC
b. Field research/lab expenses and supplies	13180000	9433263	9433263	0	71.57	
c. Operating expenses	1426620	634111	492104	142007	34.49	
d. Vehicle hire and fuel, oil & maintenance	1167266	388070	357766	30304	30.65	
e.Training/workshop/seminar	368600	206785	198600	8185	53.88	

f. Publications and printing	510000	303570	298500	5070	58.53	
g. Miscellaneous	383101	298943	298434	509	77.90	
h. Capital expenses	3954000	3843450	3843450	0	97.20	
Total	24798004	18724131	18269791	454340	73.67	

Coordination component (BARC)

Fig in Tk.

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	743913	1012178	743913	268265	100.00	Unspent money refunded to the PIU, BARC
b. Field research/lab expenses and supplies	0	0	0	0	0	
c. Operating expenses	87120	219568	86340	133228	99.10	
d. Vehicle hire and fuel, oil & maintenance	102266	132570	102266	30304	100.00	
e.Training/workshop/seminar	123600	131785	123600	8185	100.00	
f. Publications and printing	300000	303570	280000	23570	93.33	
g. Miscellaneous	63101	63139	62630	509	99.25	
h. Capital expenses	0	0	0	0	0	
Total	1420000	1862810	1398749	464061	98.50	

Component I (BFRI)

Fig in Tk.

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	407012	338390	338390	0	83.14	Unspent money refunded to the PIU, BARC
b. Field research/lab expenses and supplies	4340000	2223848	2223848	0	51.24	
c. Operating expenses	780000	291609	291609	0	37.39	
d. Vehicle hire and fuel, oil & maintenance	550000	214500	214500	0	39.00	
e.Training/workshop/seminar	245000	75000	75000	0	30.61	
f. Publications and printing	100000	0	0	0	0.00	
g. Miscellaneous	160000	150824	150824	0	94.27	
h. Capital expenses	1072500	971950	971950	0	90.62	
Total	7654512	4266121	4266121	0.00	55.73	

Component 2 (BAU)

Fig in Tk.

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
a. Contractual staff salary	2657492	2265371	2265371	0	85.24	Unspent money refunded to the
b. Field research/lab expenses and supplies	8840000	7209415	7209415	0	81.55	
c. Operating expenses	559500	122934	114155	8779	20.40	

d. Vehicle hire and fuel, oil & maintenance	515000	41000	41000	0	7.96	PIU, BARC
e.Training/workshop/seminar	0	0	0	0	0	
f. Publications and printing	110000	0	0	0	0.00	
g. Miscellaneous	160000	84980	84980	0	53.11	
h. Capital expenses	2881500	2871500	2871500	0	99.65	
Total	15723492	12595200	12586421	8779	80.05	

D. Achievement of Sub-project by objectives (Tangible form): Technology generated/developed

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible measurable)	Outcome (short term effect of the research)
1. To investigate the various environmental factors influencing the occurrence of infectious diseases of selected fin fishes and Shrimp	Various environmental factors such as temperature, Dissolved oxygen, pH and ammonia were tested on infected farms of Tilapia, V. Koi, Shing, Magur and Shrimp species.	Most of the cases poor environmental factors are responsible for disease occurrences.	Farmers were being told to maintain optimum environmental condition to their farm to reduce the occurrence of diseases.
2. To determine the pathogenicity of the field isolates of bacteria and viruses by inducing aquarium based experimental infection using different fin fishes;	The healthy individuals of selected fishes weighing 20 g were taken and acclimated at 25°C for one week in flat bottomed circular 30 L aquariums. The aquariums were filled with de-chlorinated tap water which was 70% removed daily and was also properly aerated with the help of aerators. The fish were fed a normal recommended commercial diet. Only the healthy fish which were showing normal activities selected for further experiment. *In vivo* pathogenicity test was carried out according to Keskin *et al*. (2004).	Determine the pathogenicity of the bacteria suspected to ensure etiological agents responsible for disease occurrences of fish diseases. The pathogenicity of the field isolates were tested in the laboratory condition.	Pathogenicity of *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* bacteria by experimental infection and status of antimicrobial susceptibility and resistance patterns of the organisms were done.

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible measurable)	Outcome (short term effect of the research)
3. To validate the efficacy of experimentally developed fish vaccines against five different species of fin fishes at the laboratory and at farm level.	Four Mono-valent, one Bi-valent and two Tri-valent vaccines were tested on lab and farm condition.	The efficacy of developed vaccine as well as validation process was done.	Farmers can use these vaccines to reduce the disease occurrence in their farms.
4. To isolate and identify emerging and existing viruses and bacteria responsible for mass mortality of fin fishes and Shrimp in Bangladesh	Isolation and identification of pathogenic *Streptococcus agalactiae* from Tilapia and V. Koi fishes and *Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* fromV. Koi, Shing, Magur and Shrimp	Isolation and identification of pathogenic *Streptococcus agalactiae Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* successfully completed	Isolation and identification of bacterial diseases from selected fin fishes and shrimp
	Isolation of TiLV virus from dead/moribund Tilapia fish	Isolation of TiLV virus successfully completed	Isolation and identification of viral disease from Tilapia fish
5. To detect and characterize the isolated viruses and bacteria and also to know their origin by using biological (infecting natural host system) and molecular methods (RT-PCR/PCR/nt/aa sequencing and construction of phylogenetic tree of specific genes of each isolate)	Complete biological characterization of pathogenic *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus.*	Complete biological characterization of pathogenic *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* was done successfully.	Biological characterization of pathogenic bacteria from fin fish and shrimp
	Biological and molecular characterization of TiLV	Biological and molecular characterization of TiLV did not complete yet	Biological and molecular characterization of TiLV from Tilapia fish was not done
6. To develop effective fish vaccines with the isolated viruses and bacteria against five different species of fin fishes and	Development of effective fish vaccine with the isolated *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio*	Development of effective fish vaccine with the isolated *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii*	Four Mono-valent, one Bi-valent and two Tri-valent vaccines were developed 1. Monovalent vaccine (*S. agalactiae*) 2. Monovalent vaccine (*A. hydrophila*) 3. Monovalent vaccine (*A. veronii*) 4. Monovalent vaccine (*V. parahaemolyticus*) 5. Bivalent vaccine (*A. hydrophila* + *A.*

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output (i.e. product obtained, visible measurable)	Outcome (short term effect of the research)
Shrimp prevalent in Bangladesh	*parahaemolyticus*	and *Vibrio parahaemolyticus* were successfully done	*veronii*) 6. Trivalent vaccine (*A. hydrophila* + *A. veronii* + *S. agalactiae*) 7. Trivalent vaccine (*A. hydrophila* + *A. veronii* + *V. parahaemolyticus*)
	Development of TiLV vaccine for Tilapia fish	Development of TiLV vaccine for Tilapia fish was not done	TiLV vaccine not developed
7. To evaluate efficacy of the experimentally developed fish vaccines in the specific hosts (four kinds of fin fishes) through laboratory and field trials	Efficacy of the experimentally developed fish vaccine with the *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* for Tilapia, V. Koi, Shing, Magur and Pangas	Efficacy of the experimentally developed fish vaccines were done successfully.	Efficacy of the vaccines against *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* were successfully completed in laboratory condition and field level
	Efficacy of the newly developed bacterial mono-valent, bi-valent and tri-valent vaccines and their trial for fish at the field level in five species of fishes (Tilapia, V. Koi, Shing, Magur and and Pangas)	Efficacy of the newly developed bacterial mono-valent, bi-valent and tri-valent vaccines and their trial for fish at the field level in five species of fishes (Tilapia, V. Koi, Shing, Magur and and Pangas) were done successfully.	Efficacy of the newly developed bacterial mono-valent, bi-valent and tri-valent vaccines and their laboratory trial for fish were successfully completed

E. Information/knowledge generated/policy generated

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output	Outcome (short term effect of the research)
To investigate the various environmental factors influencing the occurrence of infectious diseases of selected fin fishes and Shrimp in Bangladesh	Various environmental factors such as temperature, Dissolved oxygen, pH and ammonia were tested on infected farms of Tilapia, V. Koi Shing, Magur and Shrimp species.	Environmental factors responsible for specific fish diseases identifies	Increased knowledge of the fish culturist about environment management reduces disease occurrence.
To validate the efficacy of experimentally developed fish vaccines against five different species of fin fishes at the laboratory and at farm level.	Four Mono-valent, one Bi-valent and two Tri-valent vaccines were tested on lab and farm condition.	The efficacy of developed vaccine as well as validation process was done.	Increased income generation through increased healthy fish production
To isolate and identify	Isolation and identification	Isolation and identification	Isolation and

General/specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output	Outcome (short term effect of the research)
emerging and existing viruses and bacteria responsible for mass mortality of fin fishes and Shrimp in Bangladesh	of pathogenic *Streptococcus agalactiae* from Tilapia and V. Koi fishes and *Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus* fromV. Koi, Shing, Magur and Shrimp	of pathogenic *Streptococcus agalactiae Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus* successfully completed	identification of bacterial diseases from selected fin fishes and shrimp
To detect and characterize the isolated viruses and bacteria and also to know their origin by using biological (infecting natural host system) and molecular methods (RT-PCR/PCR/nt/aa sequencing and construction of phylogenetic tree of specific genes of each isolate)	Complete biological characterization of pathogenic *Streptococcus agalactiae,Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus*.	Complete biological characterization of pathogenic *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus* was done successfully.	Biological characterization of pathogenic bacteria from fin fish and shrimp
To develop effective fish vaccines with the isolated viruses and bacteria against five different species of fin fishes and Shrimp prevalent in Bangladesh	Development of effective fish vaccine with the isolated *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus*	Development of effective fish vaccine with the isolated *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii* and *Vibrio parahaemolyticus* were successfully done	Four Mono-valent, one Bi-valent and two Tri-valent vaccines were developed
To evaluate efficacy of the experimentally developed fish vaccines in the specific hosts (four kinds of fin fishes) through laboratory and field trials	Efficacy of the experimentally developed fish vaccine with the *Streptococcus agalactiae, Aeromonas hydrophila, Aeromonas veronii*and *Vibrio parahaemolyticus* for Tilapia, V. Koi, Shing, Magur and Pangas	Efficacy of the experimentally developed fish vaccines were done successfully.	Fish vaccines are new ready to use in farm level

F. Materials development/publication made under the sub-project

Publications	Number of publications		Remarks (e.g., paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin /booklet/leaflet/ flyer etc.	1	-	"Reduction of cultured fin fishes mortality by vaccination against infectious diseases" **Technology bulletin**
Technology bulletin /booklet/leaflet/ flyer etc.	-	1	"***Use of Fish Vaccines***" Fish Technology Fair, 2019 **Leaflet**

Publications	Number of publications		Remarks (e.g., paper title, name of journal,
Journal publications (Scientific journal)	3	1	“Isolation and molecular detection of *Streptococcus agalactiae* from popped eye disease of cultured Tilapia and Vietnamese koi fishes in Bangladesh” *J. Adv. Vet. Anim. Res.,* **8(1):** 14–23
	1		First time development and validation of the injectable and feed based bacterial vaccines against popped eye disease of cultured Tilapia and Vietnamese Koi fishes in Bangladesh. Mohummad Muklesur Rahman, Md. Ashikur Rahman, Muhammad Tofazzal Hossain, Mahbubul Pratik Siddique, Md. Enamul Haque, A. K. M. Khasruzzaman and Md.Alimul Islam **Submitted: Saudi J. Biol. Sci., Peer Review under the responsibility of King Saud University**
Conference paper (Scientific)	-	1	“First time development and evaluation of inactivated bacterial vaccine against popped eye disease of cultured Tilapia and Vietnamese Koi fishes in Bangladesh” BSVER Annual International Scientific Conference. **The 26th Conference 2020, BSVER Publication No 45, 28 February to 01 March, 2020**, Bangladesh Agricultural University, Mymensingh, pp. 69
Conference paper (Scientific)	-	1	“Investigation and characterization of viral and bacterial diseases in selected fin fishes and Shrimp in Bangladesh and development of their vaccines and validation” **Annul Workshop of BAURES Research Progress (2019-2020), pp. 35**
Conference paper (Scientific)	-	1	“Isolation, identification and molecular detection of *Streptococcus agalactiae* (GBS) as an emerging pathogen of popped eye disease of cultured Tilapia and Vietnamese koi fishes in Bangladesh” **3rd International Scientific Conference on Food Safety and Health, 1st February, 2020**
Newspaper/popular article	1	2	“বাকৃবিতে স্বাদুপানির মাছের ক্ষতিকর রোগের বিরুদ্ধে ভ্যাকসিন উদ্ভাবিত” *Amder Nuton Shomoy.* https://www.amadershomoy.com/bn/2021/06/21/1392306.asp
Other publications , if any	-	2	1.https://youtu.be/ZZxPtsh6Zxc 2.https://youtu.be/7hgwQLMQ2-c

G. Description of generated Technology/knowledge/policy

i. Technology fact sheet

Technology fact sheet: 01:*Development of vaccines and validation*

Introduction

An important role is played by aquaculture in improving for food security and economic stability. From 2000 and 2018, aquaculture production increased from 7.12 to 42.77 Lac metric tons where 44.43 percentages came from pond aquaculture. But, disease outbreaks increases with pathogens (mainly bacteria) have caused significant losses to aquaculture production and revenues (DoF, 2018). There are some species such as Tilapia (*Oreochromis niloticus*), Koi (*Anabas testudineus*), Shing (*Heteropneustesfossilis*), Magur (*Clariasbatrachus*), Pangas (*Pangasius hypophthalmus*) have great importance in aquaculture because they comprised a handsome portion in total fish production. Species composition of annual fish production of ponds in 2018-19 showed in following Table (DoF, 2018)

Sl. No.	Species	Production (MT)	%
1.	Tilapia (*O. niloticus*)	320,963	16.25
2.	Pangas (*Pangasius pangasius*)	447,054	22.64
3.	Koi (*Anabas testudineus*)	49,659	2.51
4.	Shing/Magur (*Heteropneustes fossilis/ Clarias batrachus*)	31,245	1.58

Although, Shing, Tilapia, Koi and Magur culture has great potential in Bangladesh, but unknown emerging diseases are causing serious economic losses because of their high mortality within 3 to 10 days under farming conditions. Most of the bacterial diseases which have not been detected or characterized their control/preventive measures (through vaccine) are not available yet in Bangladesh. For Biological control of fish disease this sub-project was taken jointly by BFRI, Department of Microbiology and hygiene, BAU and BARC. The aim of this research work was to develop highly effective vaccines for fishes and validate these vaccines at field level.

Description

To evaluate the efficacy of these vaccines which were produced by the BAU (component-2) of this sub-project, lab and field trials were done by BFRI (component 1). Inactivated bacterial vaccines were used in the healthy adult and brood (male and female) cultured fin fishes before starting their breeding seasons through injection at hatchery and farm levels. Two fish hatcheries and farms at Trishal and Gouripur, Mymensingh were selected for this study. The experiment was conducted in those two farms during two consecutive breeding seasons of the year 2020 and 2021. Brood of Tilapia, V. Koi, Shing and Magur were injected with that vaccines (active immunization)and the fry produced from the vaccinated fishes were fed (passive immunization) with the egg yolk of the immunized hen. During the cultured period, vaccinated fish were taken to laboratory and efficacies of vaccines were tested by determining their serum antibody level (by ELISA) and rate of protection through challenge experiment.

Suitable location/ecosystem

Mainly the hatchery owners can be the prime stakeholder and with this process the intensity of bacterial diseases can be minimised which could boost up the production and reduce the economic losses. Traditional methods (various chemicals and antibiotics) of infectious disease control of fishes are highly dangerous for fishes and human as well, the validation report on the efficacy of the newly developed vaccines can easily mitigate the loss of cultured fishes from frequent attacked of infectious diseases (bacterial and viral).

Benefit

Fish vaccines can significantly reduce specific disease-related losses resulting in a reduction of antibiotics use. The final result is the decrease of overall unit costs and more predictable production. Fish vaccines are advantageous over antibiotics because they are natural biological materials that leave no residue in the product or environment, and therefore will not induce a resistant strain of the disease organism. Across two discrete years under varying evaluation methods, vaccination resulted in significant improvements in survival, feed conversion ratio, feed fed, and total yield. These results suggest vaccination process against the disease occurring organism is an effective strategy to mitigate disease-related losses and can significantly improve production efficiency and profitability of fish culture in Bangladesh.

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Technology fact sheet: 02: *Development of bacterial vaccines for cultured fin fishes and shrimp*

Introduction

Total fish production in Bangladesh has increased six fold in the last 34 years, and fish production is now expected to reach 45.52 lakh tons by 2020–21 (FRSS, 2017). Pond culture represents the mainstay of aquaculture in Bangladesh, accounting for 85.8% of the total recorded production and 57.7% of the area under culture (DoF, 2010). The current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. However, Tilapia (*Oreochromis niloticus*), Koi (*Anabas testudineus*), Shing (*Heteropneustes fossilis*), Magur (*Clarias batrachus*) Pangas (*Pangasius hypophthalmus*) and Shrimp (*Penaeus monodon*) are being cultured in ponds mostly as commercial basis by entrepreneurial farmers in Bangladesh.

Among different freshwater fin fishes, Tilapia (*O. niloticus*), Koi (*A. testudineus*), Shing (*H. fossilis*), Magur (*C. batrachus*) Pangas (*P. hypophthalmus*) and Shrimp (*P. monodon*) are very popular and high valued fish species in Bangladesh. Although, Shing, Tilapia, Koi and Magur culture has great potential in Bangladesh but unknown emerging diseases are causing serious economic losses because of their high mortality within 3 to 10 days under farming conditions. Most of the viral and bacterial diseases which have not been detected or characterized their control/preventive measures (through vaccine) are not available yet in Bangladesh. By using different kinds of antibiotics pathogenic bacterial population of fishes are increasing day by day therefore, biological control (by vaccination) measure is getting popular all over the world today. Present research work was undertaken to develop highly effective vaccines for fishes to control their mass mortality.

Description

Inactivated bacterial vaccines can be used in the healthy adult and brood (male and female) cultured fin fishes before starting their breeding seasons through injection. Same vaccine can be injected into the healthy laying hens to develop protective level of antibodies which can be transferred through the eggs of hen. The egg yolk of the immunized hen can be fed to the newly hatched fish try for a period of two weeks at the hatchery and pond level to develop passive immunity. As fishes does not have IgG so the level of predominant IgM and IgY present in the egg yolk and the IgM which is transferred from the brood mother fish to the fry can protect fishes successfully. Biological control measure (using effective fish vaccines injectable or feed based) could be highly effective to reduce or control the infectious bacterial and viral diseases in cultured fin fishes and shrimp in Bangladesh. Following the efficacy of the newly developed injectable form of fish vaccines'feed based fish vaccines are ready and going to apply at the farm level soon to increase fish immunity through GILT system.

Suitable location/ecosystem

Most of the cultured fish farmers can be benefited by using the immunized fry of the five different species of fin fishes from the reference hatchery. Economic losses of the farmers and the ultimate losses of the country (reduce income of foreign currency) can easily be reduced by reducing mass mortality of their cultured fishes at the hatchery and ponds level. There is no potential risk or negative impacts of the newly developed fish vaccines in the human health and the environment as well. After vaccination only antibodies and the memory cell will be present in the fish body not any residue of unused antigen or adjuvant in the body of the immunized generation of the consumable adult fishes.

Benefits

The rational use of fish vaccines could be a fundamental principle of good aquaculture production and farm fish health management through prevention of microbial (bacterial and viral) diseases in Bangladesh. Fish vaccines will increase the overall survival rate of the cultured fin fishes and shrimp, which will help significant reduction of mass mortality of farm fishes at any age of their growth and also will help overall reduction in production cost. Indiscriminate uses of different kinds of antibiotics and other anti-microbial agents to control infectious diseases of cultured fin fishes can easily be omitted by the use of effective vaccine. In addition, vaccination of farm fishes will play important role for the reduction of random release of multidrug resistant bacteria in the cultured environment which is harmful to the human health.

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ii. Effectiveness in policy support(if applicable)

Findings of the research will be working as supportive information in favour of existing aquaculture water bodies environment management related to fish disease control regulations/acts and necessary amendments, where applicable.

H. Technology/knowledge generation/policy support (as applied)

i. Immediate impact on generated technology (commodity and non-commodity)

To reduce the prevalence of diseases, farmers generally required to maintain optimal environmental conditions at their farms. They could use highly effective newly developed biological method (vaccination)to reduce early mortality and increase productivity of fin fish and shrimp at the hatchery and in pond aquaculture.

ii. Generation of new knowledge that help in developing more technology in future

The benefits of innovation of the development of fish vaccines might be helpful to the individuals, communities, industries, societies, nations, regions, and the world those are directly or indirectly involved in aquaculture system. There is ample scope to develop better preparation of fish vaccines (regular feed based) against any kind of pathogenic bacterial and viral diseases using nano-technology in future which will provides more opportunities for increase productivity of antibiotic residue and disease-free healthy fishes as safe protein for human food chain.

iii. Technology transferred that help increased agricultural productivity and farmers' incomes: ***Not applicable at this stage.***

iv. Policy support: *Not applicable.*

I. Information regarding desk and field mentoring

i. Desk monitoring (description and output of consultation meeting, monitoring workshops/seminars etc.)

Date of the programs	Program descriptions	Implementation Unit	Remarks/output
24 December, 2017	Signing of the letter of agreement (LoA)	PIU-BARC, NATP-2	Duly signed as per the terms and condition of LoA
06 November, 2018	Inception workshop of PBRG sub-project ID: 030	Fisheries Division, BARC	The meeting was to inform the PIs of the sub-project how follow the PCR 2008 for the recruitment of manpower and procurement of equipment’s, chemicals and reagents as per budget.
17 June, 2019	Monitoring workshop on PBRG sub-project ID: 030	PIU-BARC, NATP-2	Progress of procurements of equipment’s, chemicals and reagents and research activities
20 July, 2019	Financial management workshop	PIU-BARC, NATP-2	Procurement of equipment’s , chemicals and reagents as per the rule of PPR 2008 and the audit rules
14 October, 2019	Coordination meeting	Fisheries Division, BARC	Discussion among the coordinator, director, PIs and Co-PIs about the guidelines how to achieve the goal collaboratively
20 November, 2019	Annual progress review workshop	PIU-BARC, NATP-2	Presentation on annual research progress through multimedia presentation. Received review comments on progress and future works
29 January, 2020	Half yearly progress review workshop	Fisheries Division, BARC	Presentation on half yearly research progress through multimedia presentation and all the suggestions were complied
25 June, 2020	Half yearly review workshop	Fisheries Division, BARC	Presentation of research progress through multimedia presentation. Received review comments.
7 September, 2020	Virtual Meeting on Progress Monitoring of PBRG Sub-projects	PIU-BARC, NATP-2	Presentation of research progress through multimedia presentation. Received review comments.
23 September, 2020	Annual progress review workshop	PIU-BARC, NATP-2	Presentation on annual research progress through multimedia presentation and the suggestions from the workshop were complied.
24 October, 2020	Annual progress review workshop	PIU-BARC, NATP-2	Presentation on annual research progress through multimedia presentation and the suggestions from the workshop were complied.
22 November, 2020	Inaugural Session of Annual Review Workshop on PBRG Sub-projects	PIU-BARC, NATP-2	Honourable Minister, MOA inaugurated and the progress of research project was through virtual multimedia presentation and the suggestions from the workshop were complied.

Date of the programs	Program descriptions	Implementation Unit	Remarks/output
29 December, 2020	Preparation process of Project Completion Reports (PCR) of PBRG Fisheries Sub Projects	Fisheries Division, BARC	Detail discussion was on the guidelines how to write and submit the draft of PCR of the project in time. Discussion was very effective
17 June, 2021	Virtual Meeting on Progress Monitoring of PBRG Sub-projects	PIU, NATP-2, BARC	Further discussion on submission of technology fact sheet and submission of PCR in time

ii. Field monitoring

Visit no.	Monitoring team	Date(s) of visit	Output
1.	1. Dr. Mian Sayeed Hassan, Director, PIU-BARC, NATP-2 2. Dr. Nowsher Ali Sarder, Monitoring and Evaluation Specialist, PIU-BARC, NATP-2	17 February, 2019	Visited research field at Trishal & BAU Microbiology laboratory; Advices helped to improve research management
2.	1. Dr. ASM Anawarul Haque, Member Director (Admin. & Finance), BARC, Dhaka. 2. Dr. Md. Mosharraf Uddin Molla, CSO (IRS), BARC, Dhaka. 3. Mr. Ajidt Kumar Chakroborty, Director (Finance), BARC, Dhaka. 4. Mr. Dipok Kumar, Monitoring Associate, PIU-BARC, NATP-2, Dhaka.	27 March, 2019	Monitored research field & laboratory; some advices were given; action was taken accordingly.
3.	Dr.Md. Harunur Rashid, Director, PIU-BARC, NATP-2	17 June, 2019	Visited research field & laboratory; some advices were given; action was taken as per review comments of local meeting.
4.	Dr. Md. Monirul Islam, Member Director (Fisheries), BARC	3 September, 2019	Visited research sites and laboratory. Instructions given by the Coordinator was executed.
5.	Atsushi Yamamoto Professor (Fish Pathology) Faculty of Fishries, Kagoshima University Kagoshima, Japan	5 September, 2019	Visited research field & laboratory; some advices were given and inspired to complete the research successfully.
6.	1. Dr. Md. Abdul Jalil Bhuyan, Research Management Specialist 2. Munshi Mamunur Rahman. Documentation Associate. PIU-BARC, NATP-2	14 December,2019	Visits research sites and laboratory. Advised to carry out researches perfectly and management of data records;
7.	Dr. Saleh Uddin Ahammed Consultant, PBRG sub-project, BARC	24 Dec'19;15 July'20	Visited research field & laboratory. Inspired to complete vaccine trials as per schedule;
8.	Dr. Md. Harunur Rashid, Director, PIU-BARC, NATP-2	22 August, 2020	Visited research field & laboratory; some advices were given; action was taken accordingly.

Visit no.	Monitoring team	Date(s) of visit	Output
9.	Dr. Mian Sayeed Hassan, Director, PIU-BARC, NATP-2	24 October, 2020	Visited research field & laboratory; some advices were given; action was taken accordingly.
10.	Dr. Saleh Uddin Ahammed Consultant, PBRG sub-project, BARC	03 November, 2020	Visited research field & laboratory. Advices helped to improve research management
11.	1. Dr. Md.Harunur Rashid, Director, PIU-BARC, NATP-2 2. Dr.Nowsher Ali Sarder, Monitoring and Evaluation Specialist, PIU-BARC, NATP-2	17 May 2021	Visited research field & laboratory; Urged and inspired to complete vaccine trials as per schedule; Action was taken accordingly.

Plate 36. A coordination meeting of PBRG sub-project held at BAU campus on 17-02-2019.

Plate 37. A monitoring team of PBRG sub-project at BAU on 27.03.2019.

Plate 38. Sub-project Coordinator & MD Fisheries Divi. Dr. Md. Monirul Islam & Consultant Dr, Saleh Uddin A, Visited BAU ON 03.09.2019 & 24.12. 20 respectively.

iii. Weather data, flood/salinity/drought level (if applicable) and natural calamities

Parameters	Seasons						Remarks
	Pre-Monson (Jan – Apr)		Monson (May – Aug)		Post Monson (Sept – Dec)		
	Max	Min	Max	Min	Max	Min	
Av. Rainfall (mm)	592	68	948	450	138	44	
Av. Temperature (^{O}C)	30	24	32	28	29	22	
Av. Humidity (%)	56	44	89	78	40	30	
Flood (year & category)	Not applicable for the area						
Av. Salinity (ppt)	0.8 – 1.2						
Drought	Not reportable draught occurred during the period.						
Natural calamity (Frequency & category)	Not reportable natural calamities occurred during the period.						

Three years average weather information of Mymensing region (2018 -20), BBS (2020)

J. Sub-project Auditing (covers all types of audites performed)

Coordination component

Types of audits	Major observation/ issues/objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial & Performance Audit by FAPAD on 31.10.19 for the year 2018-2019	No objection raised, found all relevant documents updated as per guideline	57825.00	Financial management of the component found running smoothly till the end of the sub-project. No	Financial management & sub-project performance found satisfactory

Financial & Performance Audit by FAPAD on 09.12.20 for the year 2019-2020.	No objection raised, found all relevant documents updated as per guideline	410131.00	query or objection raised at any stage of operation by the audit teams.	Financial management & sub-project performance found satisfactory
Financial & Performance Audit by FAPAD on 11.10.20 for the year 2020-2021.	No objection raised, found all relevant documents updated as per guideline	400650.00		Financial management & sub-project performance found satisfactory

Component-1 (BFRI)

Types of audit	Major observation /issues/ objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial & Performance Audit by FAPAD 20-11-2019	No objection raised, found all relevant documents updated as per guideline	1035474.00	Financial management of the component found running smoothly till the end of the sub-project. No query or objection raised at any stage of operation by the audit teams	Financial management found satisfactory
Financial audit by M.l. Chowdhury & Co. 17-12-20	No objection raised, Found all relevant documents updated as per guideline	1621308.00		Financial management found satisfactory
Financial & Performance Audit by FAPAD 28-10-2021	No objection raised, Found all relevant documents updated as per guideline	42462I.00		Financial management found satisfactory

Component-2 (BAU)

Types of audits	Major observation/ issues/ objections raised; if any	Amount of Audit (Tk.)	Status at the sub-project end	Remarks
Financial & Performance Audit by FAPAD 30-09-2019	No objection raised, found all relevant documents updated as per guideline	6881616.00	Financial management of the component found running smoothly till the end of the sub-project. No query or objection raised at any stage of operation by the audit teams	Financial management found satisfactory
Financial audit by M.l. Chowdhury & Co. 20-12-20	No objection raised, found all relevant documents updated as per guideline	3416313.00		Financial management found satisfactory
Financial & Performance Audit by FAPAD 28-I0-2021	No objection raised, found all relevant documents updated as per guideline	22879I7.00		Financial management found satisfactory

K. Lessons Learned

From the findings of the present study it is learned that the physico-chemical properties of culture environment greatly influences disease occurrence and the use of fish vaccine (either injectable or feed based) could be the best alternative to maintain health and to increase growth and productivity of cultured fishes against infectious diseases (bacterial and viruses) rather than the use of antibiotic and different chemical agents.

L. Challenges

Due to COVID-19 pandemic situation it was very difficult to start the field research for the validation of efficacy of the newly developed fish vaccines in time. Thuswe failed to procure important chemicals/equipment etc. for the development fish vaccines. Under this unusual circumstance, only injectable form of bacterial vaccines (mono-valent, bi-valent and tri-valent) were developed and their efficacy were validated but the development of viral vaccines (TiLV) could not attained.

M. Suggestions for Future Planning

Bacterial and viral diseases of all the high valued and consumable species of cultured fin fishes and shrimp must be brought under vaccination during any outbreak responsible for high mortality which might help better management of their health and also help to increase their productivity. Biological control methods (vaccination) of fish diseases could be the good/ standard method than the other traditional methods (chemical and antibiotics) in modern farming of fishes in Bangladesh. Therefore, future thrust should be given on more research on this area that should be a continuous process.

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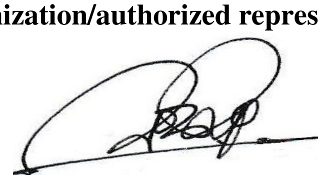
Signature of the Coordinator	**Counter signature of the Head of the organization/authorized representative**
 **(Dr. Md. Monirul Islam)** Date: 15.10.2021 ***Member Director (Fisheries)*** ***Bangladesh Agricultural Research Council***	 **(Dr. Shaikh Mohammad Bokhtiar)** Date: 15.10.2021 ***Executive Chairman*** ***Bangladesh Agricultural Research Council***

APPENDICES

Appendix – BARC: A: Recommendation of the inception workshop and status of action taken

Coordination component (BARC)

Recommendations	Action taken
General recommendation	
Revision of the title of the sub-project suggested	Titled revised as follows: "Investigation and characterization of viral and bacterial diseases in selected fin fish and shrimp in Bangladesh, vaccines development and validation"
Component 1 (BFRI)	
• Immediate placement of an experience scientist of respective field as PI of the component sub-project urges	Proposal has been placed by the respective division of BARC and action taken by the respective institute later.
• Appropriate reflection of outputs and outcomes of the component sub-project suggested	Done as per suggestion
Component 2 (BAU)	
• No comment made	

Appendix – BARC: B: Recommendation of the half yearly workshops

Recommendations of the First Half Yearly Workshop	Action taken
Component 1 (BFRI)	
• Water quality parameters and their relationship with the disease occurrence need to be focused more clearly	Work covered under existing objectives
• Immediate completion of histological works and other follow-up related study for vaccine development and validation should be done without any further delay. The time limit of the sub-project should be taken into consideration seriously in this regard	The progress is per sub-project activity workplan. All recommended activities are there under the planned list of activities
Component 2 (BAU)	
• Clear results of vaccine trials not focused in the presentation	Vaccine trial results are shown after completion of lab & field level trials
• Further efforts on various vaccine production (as per objectives) and their validation emphasized to complete the research within the stipulated timeframe of the sub-project	Majority work done within the stipulated time. Outbreak of Covid-19 hampered due progress of the research. However, the remaining works done later as per guideline of the coordination unit as per plan

Appendix – BARC: C: Recommendation of the annual workshop

Recommendations of the First Annual Workshop	**Action taken**
Component 1 (BFRI)	
• Baseline study data/information was not presented	Baseline study report completed and was presented in the second half yearly review workshop
• Information/results on environmental parameters study (as per objective 1) was not shown in the presentation	Data on environment presented in the second half yearly review workshop
• Suggested to present quantity of sample as % of total populations instead of mentioning numerical figures	Necessary correction done
• Importance given on use of advance method (instead of plate count) to find out the most accurate number/conc. of bacteria in samples considering the unit "MPN".	Recommendation adapted
Component 2(BAU)	
• Baseline study data/information was not presented	Baseline study report completed and was presented in the second half yearly review workshop
• Residual effect of antibiotics and vaccine must be taken into attention in case of their application (if any) in fish body/culture system;	Necessary study carried out
• Preference of oral administer of vaccine as cost effective and efficient method for fish disease treatment suggested;	Oral administer of vaccine as cost effective and efficient method for fish disease done and findings presented.
Recommendations of the second annual workshop	
Component 1 (BFRI)	
• No comment	
Component 2 (BAU)	
• Evaluation of the efficacy of the experimentally developed vaccines in specific host is yet to complete. The PI will take necessary attempt to do the needful for validation of vaccines and side by side trials to administer oral based vaccinated feed to be completed.	Efficacy of the experimentally developed vaccines in specific host, validation of vaccines and trials to administer oral based vaccinated feed completed about 90%.
• PI should strictly follow the expenditure of sub-project money as per approved plan. In case of any expenditure made which is not included in the research plan (such as purchase of poultry feed etc), the PI will be liable for any sort of problem, if arises;	Necessary action taken

Appendix – BARC: D: Recommendation of the coordination meetings

Central coordination meeting at BARC	
Recommendations	**Action taken**
• Long-time absence of the PI of the BFRI component creates stagnancy of the research activities in the part which as whole affected the total sub-project progress by creating research gap between the components. Therefore, it is suggested to arrange frequent discussion session and meetings with the newly appointed PI of the BFRI component to resolve the situation. The PI of the BAU part is requested to take initiative in this regard;	Necessary action taken
Two Other virtual coordination meetings	
Component 1 & 2 ID 030 (BFRI& BAU)	
• As both the components (BFRI and BAU) has been working simultaneously with the general objective of developing effective fish vaccines against selective bacterial and viral diseases in fin fish and shrimp, therefore, they have to maintain effective coordination to achieve the target of acceptable fish vaccine development. The steps of working chain to be maintained in this regard by the components are as follows: a. Study and impact analysis of environmental factors/ water quality etc. as causing agent of fish/shrimp diseases. b. Collection of different morbid fish/shrimp samples. c. Culture and pathogenicity test of microbe samples at laboratory level. d. Molecular identification/characterization, PCR test and initial formulation of 4 microbe vaccine. e. Primary efficiency test of the microbe vaccines at laboratory. f. Injected first dose of the selected vaccine to the body muscle of sorted out broods of different species (Tilapia. Koi, Shing, Magur) at newly selected hatchery complex. g. Based on necessity, injected second dose of vaccine after 10 days of first injection to the same species of fish. h. Parallel activity to inject inter muscular microbe vaccine to the muscles of layer hens. i. Induced breeding and production of fish spawn of the injected broods. j. Allow spawn to feeding started (from the first day of mouth feeding) with layer egg yolk and milk caster for microbe vaccine property transmission. k. Allow spawns to attain fingerlings size within a period of 30-40 days under an application of feed-based microbe vaccine (at 2nd and 7th day); l. Rearing fingerlings (age 2-2.5 months) using feed-based vaccine (feeding 5 days with vaccinated feed); m. Laboratory challenge testing of cultured samples for proficiency and effectiveness of the microbe vaccines for disease controlling selective fish species after 15 – 20 days	Recommended steps were followed by both the components and achieved satisfactory level of output.

of feed vaccine application.

n. Confirmation of vaccine formulation and development through various trials and error.

o. Characterization and preliminary development of Tilapia Lake Virus, efficient test and validation/challenge test will be completed within September/October.

p. Study on toxic control and antigen development in shrimp body will continue from August –November.

- More study for supporting baseline information generation on environmental factors those influencing occurrence of infectious disease in fish and shrimp with various research observation are to be added ***by component 1 (BFRI)*** to enrich the baseline report as a part of final report.
- Close observation on primarily successfully tested samples of microbe vaccine (Trivalent-2 sp; Divalent-1 sp and Monovalent-2 sp) recently applied in brood body on last 17 June'20 should keep under close observation of fish behaviour before validation test at BFRI laboratory again (Species list are shown under table A).

Table A

Douhakhola

Species	Vaccine	Dose
Magur (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi* *Vibiro parahaemolyticus*	.5 ml
Magur (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi* *Streptococcous agalactiae*	.5 ml
Koi (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi* *Vibiro parahaemolyticus*	.5 ml
Koi (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi* *Streptococcous agalactiae*	.5 ml
Shing (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi*	.5 ml
Shing	*Aeromonas hydrophila*	.5 ml
Shing	*Aeromonas vironi*	.5 ml

Trisal

Species	Vaccine	Dose
Tilapia (10 ♀ + 5 ♂)	*Aeromonas hydrophila* *Aeromonas vironi* *Streptococcous agalactiae*	.5 ml

Recommended steps were followed by both the components and achieved satisfactory level of output.

Component 2 (BAU)
Appendix 1

Composition of media

Media Name	Composition	g/L
Nutrient broth	Peptone	5.0
	Sodium chloride	5.0
	Beef extract	1.5
	Yeast extract	1.5
	Final pH (at 25° C)	7.4±0.2
Alkaline Peptone Water (APW)	Peptone	10.0
	Sodium chloride	10.0
	Final pH (at 25° C)	8.4±0.2
Mueller Hinton (MH) agar	Beef, dehydrated infusion	300.0
	Casein acid hydrolysate	17.5
	Starch	1.5
	Agar	15.0
	Final pH (at 25° C)	7.3±0.1
Blood Agar Base (BAB)	Meat Extract	10.0
	Tryptose	10.0
	Sodium Chloride	5.0
	Agar	15.0
	Final pH (at 25° C)	7.3±0.2
Thiosulfate-citrate-bile salts-sucrose (TCBS) Agar	Yeast Extract	5.0
	Peptone	10.0
	Sodium Citrate	10.0
	Sodium Thiosulfate	10.0
	Ox-gall	8.0
	Sucrose	20.0
	Sodium Chloride	10.0
	Ferric Citrate	1.0
	Thymol Blue	0.04
	Bromothymol Blue	0.04
	Agar	15.0
	Final pH (at 25° C)	8.6±0.2
Tryptic Soy Agar (TSA)	Pancreatic digest of casein	15.0gm
	Peptic digest of soybean meal	5.0gm
	Sodium chloride	5.0gm

	Agar	15.0gm
	Distilled water	1 liter
	Final pH (at 25°C)	7.3±0.2
Media Name	**Composition**	**g/L**
Tryptic Soy Broth (TSB)	Tryptone (Pancreatic Digest of Casein)	17.0 g
	Soytone (Peptic Digest of Soybean)	3.0
	Glucose (= Dextrose)	2.5
	Sodium Chloride	5.0
	Dipotassium Phosphate	2.5
	Final pH (at 25°C)	7.3±0.2

Montanide™ ISA

These oil-based adjuvants are intended for use inwater-in-oil (W/O), oil-in-water (O/W) or water-in-oil-in-water (W/O/W) emulsions. Composed of mineral oil, non-mineral oil, or a composition of both, as well as surfactants, these adjuvants are highly robust and ready to emulsify.

Appendix 2

1. Composition of different reagents: Phosphate buffer saline

Composition	Unit
Sodium chloride	8.0 gm
Disodium hydrogen phosphate	2.8 gm
Potassium chloride	0.2 gm
Potassium hydrogen phosphate	0.2 gm
Distilled water to make	1000 ml

2. Composition of PBS (1× X)

Salt	Concentration (mmol/L)	Concentration (g/L)
NaCl	137	8.0
KCl	2.7	0.2
Na_2HPO_4	10	1.42
KH_2PO_4	1.8	0.24

Start with 800 mL of distilled water to dissolve all salts. Add distilled water to a total volume of 1 liter. The resultant 1× PBS will have a final concentration of 157 mM Na^+, 140mM Cl^-, 4.45mM K^+, 10.1 mM HPO_4^{2-}, 1.76 mM $H_2PO_4^-$ and a pH of 7.96. Add 2.84mM of HCl to shift the buffer to 7.3 mM HPO_4^{2-} and 4.6 mM $H_2PO_4^-$ for a final pH of 7.4 and a Cl^- concentration of 142 mM.

3. Kovac's reagent for Indole preparation

Composition	Unit
P-dimethyl aminobenzaldehyde	5.0 gm
Amyl alcohol	75.0 gm
Conc. HCL	25 gm

4. V-P reagent-1

Composition	Unit
Ethyl alcohol	5 %

5. V-P reagent-2

Composition	Unit
Creatinine	0.3%
Cotton blue	0.05 m

6. Methyl red solution

Composition	Unit
Methyl red	0.05 gm
Ethanol (absolute)	28 ml
Distilled water	22 ml

7. Phenol red solution

Composition	Unit
Phenol red	0.2 gm
Distilled water	100 ml

8. Calculation of protein concentration (C = A/(L x Ɛ))

A	Absorbance (λ= 450 nm)
ε	Molar Absorptivity of the absorbing species
L	length of light path (cm.)
C	Concentration (mg/ml)

9. Solutions for Gram's staining

a. Stock Crystal	**Composition**	**Unit**
Violet solution	Ethyl alcohol	10.0 gm
	Acetone	1000 ml

b. Stock oxalate	**Composition**	**Unit**
Solution	Ammonium oxalate	1.0 gm
	Distilled water	1000 ml

c. Stock violate Working solution	Composition	Unit
	Stock Crystal violet solution	20 ml
	Stock oxalate solution	80 ml

d. Gram's Iodine solution	Composition	Unit
	Iodine crystal	1.0 gm
	Potassium Iodide	2.0 gm
	Distilled water	300 ml

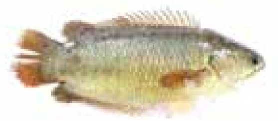
e. Acetone alcohol	Composition	Unit
	Ethyl alcohol	250 ml
	Acetone	250 ml

f. Safranin working solution	Composition	Unit
	Safranin	2.5 gm
	Ethyl alcohol (95%)	100 ml
	Distilled water	300 ml

Appendix 3

The important instrument and apparatus used through the study are listed below:

Instruments used	Company name
Electric Balance (Type-AY220)	Shimadzu Corporation, Japan
Micropipettes	Global in vitro LLP, UK
Microwave Oven	Binder, USA
Refrigerator (SL.No.209NRCQ074287)	LG, India
Incubator (Model:VS-1203P3V)	Nanotech Co. Ltd, Korea
Vortex Mixture, VM	Taiwan
Stream Sterilizer	Japan
Laminar Airflow (Model: PCR4-A1)	Korea
Biosafety Cabinet (Class-2)	China
Autoclave (Model:DAC-45) wise clave	Human lab, Korea
Beaker	Polylab products
Micropipette	Global invitro LLP,UK
Microscope (Model:BM-180/sp)	Boeco, Germany .
Test Tube	Polylab products
Petri plate	Polylab products
pH & temperature meter	AD 1000
Shaking Incubator (Model WIS-20)	Daihan Scientific Co, Ltd., Korea
Needles and Loops	HiMedia Lab. Pvt., Ltd
Ultra-low temperature freeze (-86^{0}C)	Japan

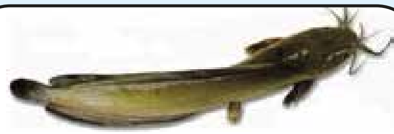


Vietnamese koi
(Anabas testudineus)

Pangas
(Pangasius hypophthalmus)

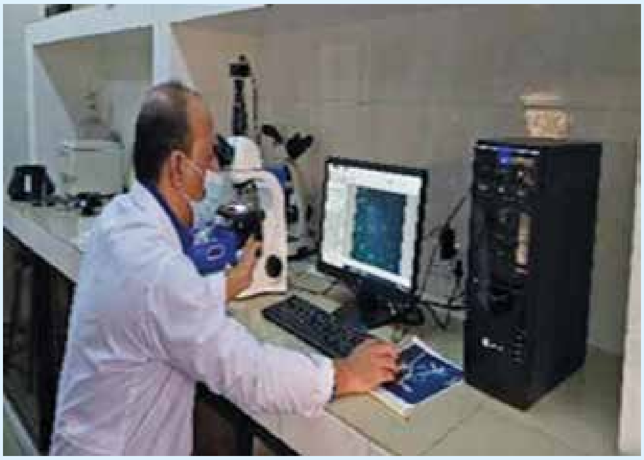
Tilapia
(Oreochromis niloticus)

Shrimp
(Penaeus monodon)



Magur
(Clarias batrachus)

Shing
(Heteropneustes fossilis)



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