PROPHYLAXIS IN AQUACULTURE

Edited by

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Cover Photo : Mangrove Red Snapper, *Lutjanus argentimaculatus*

Foreword

World fish production reached 167.2 million tonnes in the year 2014. The capture fisheries being stagnant from the late 1980s, aquaculture has performed impressively and has contributed 73.8 million tonnes to the total production. A milestone was reached in 2014 when the aquaculture sector's contribution to the supply of fish for human consumption overtook that of wild-caught fish for the first time. World aquaculture production has increased by about 33% in the last five years. Thirty five countries, including India, produced more farmed fish than wild-caught fish. All this became feasible due to the progressive intensification of aquaculture, using scientific and technological backstopping, in the areas of seed, feed, genetics and health.

Increased production due to intensification also had a price to pay. Intensive aquaculture with high stocking density has resulted in increased incidence of diseases threatening the sustainability of aquaculture. Diseases such as white spot syndrome and vibriosis of shrimps, viral nervous necrosis and argulosis of finfishes continue to cause considerable damage to the industry and yet no foolproof control measures are available. To add to the woes, new and emerging diseases like *Enterocytozoon hepatopenaei* (EHP) are being reported. With the globalisation of trade and international movement of live fish, fertilized eggs and feed, it is tough to prevent the introduction of exotic pathogens.

A multi-pronged approach to control the incidence and introduction of diseases in aquaculture is essential. Better health management through the application of probiotics and immunostimulants, selective breeding to produce disease-resistant stocks, improved feeds and feeding methods, adopting strict biosecurity protocols, quarantine and screening of imported live fish and feed and vaccination of susceptible stock are some of the ways to prevent diseases in cultured species. Many research activities have been initiated in these areas in the country and many significant leads have been established. In the Indian scenario, information available in these areas are scattered and need compilation to come up with a single source of information for the aquaculture professionals. The compendium on 'Prophylaxis in Aquaculture', prepared under the consortium research platform on vaccines and diagnostics by scientists of CIBA and other partnering institutes, is a collection of information on various aspects of prophylaxis in aquaculture and will be a useful reference material for all researchers, students and stakeholders.

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Contents

I. General Aspects

Scope of prophylactics in aquaculture

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In the face of global climate change, economic and financial uncertainties and the competition for natural resources, we have a challenge to feed 9 billion people by 2050. Fisheries and aquaculture have the potential to feed the growing population utilizing the natural resources to achieve food security and ensure economic and social development. Capture fisheries production is static since the late 1980s and for the first time in 2014 the contribution from aquaculture has exceeded that of capture fisheries. Global supply of fish for human consumption is growing at 3.2%, reaching an estimated global per capita consumption of 20 kg. In the year 2014, of the total 93.4 million tonnes of global capture fish [1], 81.5 million tonnes was from marine waters and 11.9 million tonnes was from inland waters [2]. During the same year the world aquaculture production of 73.8 million tonnes comprised of 49.8 million tonnes of finfish, 16.1 million tonnes of molluscs, 6.9 million tonnes of crustaceans and 7.3 million tonnes of other aquatic of animals. Aquaculture contributed 44.1% of the total fish production. At the top of the world marine fish catch, China produced more than 60% of cultured fish during 2014. Thirtyfive countries including India, China, Viet Nam, Bangladesh and Egypt, produced more fish from culture than from the wild [2].

The health benefits of seafood and the increasing purchasing power of the people from the developed and developing countries have led to improved demand for fish and fisheries products. Since the South East Asian countries are bestowed with suitable climatic conditions for aquaculture, this region contributes more than 90% of world aquaculture production. During the year 2015-16 India exported 0.95 million tonnes of marine products worth USD 4.7 billion which includes 0.37 million tonnes of shrimp worth USD 3.1 billion. Interestingly, the major exports during this period went to South East Asia followed by the European Union, the USA, Japan and China [3].

Aquaculture is defined as "the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants" [4]. Aqua farming includes regular stocking, feeding and protecting the animals from environmental stress and infectious agents to maximize the production and productivity. With growing competition in the international market and decreasing natural resources, intensification is being emphasized as the possible option for the economic sustainability of the industry. There is an explosive growth of the industry due to research and development in the different areas of aquaculture. Newer technologies are being demonstrated for improved productivity along with environmental safety.

Maintaining good health of aquatic animals is the key to the economic sustainability of the industry. Losses due to diseases and environmental stress have become a major impediment in the economic sustainability of the industry. Prophylactics and therapeutics are the major approaches for effective health management both in human and veterinary medicine. Application of drugs and medicines has become inevitable to maintain the health of the cultured animals and the environment. Prophylactic approach in aquaculture will reduce our dependence on antimicrobials, disinfectants, anti-parasitic drugs which are known to be harmful to the host and the environment in the long run. Due to the nature of aquaculture systems and the possible environmental impacts, treating of disease conditions using chemical or biological agents is not practical. Protecting the culture environment from deteriorating and cultured animals from falling sick is the best possible option for production of healthy aquatic animals. Till the development of antibiotics in the early 19th century the human race was almost entirely dependent on the prevention of the disease rather than treatment. The importance of prophylactics in human history could be assessed by detailed descriptions available in the ancient Indian and Chinese medical literatures.

General prophylactic measures followed in aquaculture operations

(a) Better management practices (BMPs) for optimum culture environments

(b) Practising microbial bioremediation using probiotics

(c) Enhancing the general disease resistance by immunostimulants

(d) Immunization of the host against specific pathogens using vaccines

(e) Nutritional interventions using nutraceuticals

(f) Selective breeding for genetic resistance

Better management practices (BMPs): Intensification in aquaculture leads to the accummulation of metabolites and uneaten feed in the pond bottom causing degradation of the culture environment, putting stress on the environment and the cultured animals. It is essential to maintain the optimum water and soil quality parameters like, pH, transparency, hardness, ammonia, nitrite, nitrate, sulphur, etc. Better Management Practices (BMPs) have been suggested for the hatchery and grow-out cultures to maintain optimal environmental parameters.

Probiotics: In nature micro-organisms play an important role in balancing between the living creatures and the environment by recycling natural resources. The utility of microbes in improving the health of the host and the environment has been investigated intensively in recent times. Microbes in the form of probiotics have been extensively

used in medicine, animal husbandry and agriculture worldwide. Probiotics are being used in human medicine from neonatal diarrhoea to mental disorders [5]. Similarly, the microbes are being used for environmental protection from septic tanks to oil wells. In poultry industry probiotics are used extensively to improve growth, intestinal microbiota and intestinal morphology, immunity, egg production and meat quality [6]. In animal husbandry, probiotics are being used both as therapeutics for the treatment of diarrhoea and as prophylactic to improve growth and milk production [7]. Plant growth promoting rhizobacteria (PGPR) are the beneficial microbes known to increase agriculture crop yields by regulating hormonal and nutritional balance, inducing resistance against plant pathogens, solubilize nutrients for easy uptake by plants and antagonize pathogens [8]. In aquaculture, probiotics have been reported to improve growth, immunity, water quality, nutrient digestibility, stress tolerance and reproductive performance in addition to inhibition of pathogens [9-11].

Microbes through the process of bioremediation have been extensively used to clean up environmental toxic pollutants. These beneficial bacteria possess the ability to degrade, transform or chelate various toxic chemicals and hence remove contaminants like heavy metals, metalloids, radioactive waste and oil products [12, 13]. Additionally, these microbes have also been reported to remove toxins from agriculture [14] and marine environments [15, 16]. Similarly, the role of ammonia oxidizing bacteria (AOBs), nitrite oxidizing bacteria (NOBs), denitrifying bacteria (DNBs), sulphur oxidizing bacteria (SOBs) and sulphur reducing bacteria (SRBs) in mitigating the toxic build-ups in aquaculture environments have been well documented. The enormous power of microbes can be exploited for improving the health and production of cultured aquatic organisms.

Immunostimulants: Enhancing the disease resistance power of the host is one of the important approaches for preventing diseases. Several substances of natural, chemical and microbial origins have been known to possess immune stimulating potential in both humans and animals. Though the administration of immune stimulating agents for general health and wellbeing of humans and animals is well established, its importance has been recognized only recently in aquaculture. Development of immune stimulants for use in aquatic organisms was slow due to the lack of understanding of basic immunology and the efficient parameters to evaluate the immune response in crustaceans and molluscs. Recent surges in the information on the molecular immune system of aquatic invertebrates and tools to evaluate the immune reactive molecules have made it possible to develop new molecules for effective stimulation of the immune system of aquatic organisms and corresponding resistance to invading pathogens.

Vaccines: Development of vaccines is considered the most significant medical achievement in human history after antibiotics which paved the way for the prophylactic in health care systems. Vaccination is being implemented both for human and animals

as national programs at international levels by leading organizations like WHO, FAO, OIE etc. Eradication of small pox in humans and the deadly cattle plague (Rinderpest) in animals was possible only due to the development of effective vaccines. Presently, there are about 20 human diseases and similar number of animal diseases for which vaccination is regularly practised worldwide. As per the latest report (4th October 2016) USDA has approved 141 vaccines and 74 bacterins for use in veterinary practice while there were only nine such products approved for use in aquatic animals as on 30th August 2016. This suggests the need for developing vaccines for aquatic animals. Lack of vaccines for aquatic animals could be attributed to the lack of effective delivery systems. Recently, several organizations have developed mineral oil and nanoparticle-based delivery methods for use in aquatic animals. As the vaccine delivery through oral, injectable and immersion means is being standardized, it should be possible to develop vaccines for different species of finfish and shellfishes in the near future.

Though research on development of vaccines for fishes is limited, the success of the vaccination program introduced in Atlantic salmon during the 1980s in Norway in reducing the use of antibiotics has proved the potential of vaccine in aquaculture [17]. Following the introduction of a vaccination program in Norwegian salmon farming, the annual consumption of antibiotics has come down from 50 metric tonnes in 1987 to 746.5 kg in 1997. During the same period, an increase of 53,000 to 353,000 metric tonnes in the farmed finfish production was recorded [18].

Nutraceuticals : According to Stephen De Felice, founder chairman of Foundation for Innovation in Medicine (FIM), who coined the term "nutraceutical", defined it as "a food (or a part of food) that provides medical or health benefits, including the prevention and or treatment of a disease", they include herbal/ natural products, dietary supplements and functional foods. Nutraceuticals gained importance in the last one decade due to the increasing cost and side effects of therapeutic pharmaceutical agents. This concept of food for health and wellbeing was the main basis of the Indian system of medicine for ages which is gaining importance following the drawbacks of modern medical systems. Stress is the common factor affecting the humans (due to modern lifestyle) and plants and animals (due to farm intensification). These groups of products containing dietary fibre, prebiotics, probiotics, polyunsaturated fatty acids, antioxidants and other different types of herbal/ natural foods primarily improve the antioxidant defence mechanism and innate immunity. Though the beneficial effects of nutraceuticals have been well established in human and veterinary medicine, similar reports are limited in aquaculture.

Genetic selection: Diseases are becoming a major hurdle in the economic sustainability of agriculture, including livestock and aquaculture. New diseases are emerging due to the increased culture intensity, climate change and crossing over of pathogens between

species. Ever increasing cost of disease prevention and control has shifted the focus of health management from prophylactic and therapeutic intervention to genetic selection. Genetic selection for disease resistance has been the milestone in the field of agriculture to meet the global hunger for food. Breeding for disease resistance is a well-established science in the field of crop protection and has shown mixed success in the livestock sector. However such programs for cultured aquatic species are limited.

Since the genetic selection programs are time-consuming, involving huge costs, the important factor to be considered is the economic cost of the disease in question. Selection for production traits can be easily achieved by measuring the growth and survival parameters. However, the selection for disease resistance is complicated by the possibility of increasing susceptibility to non-target pathogen and the loss of production traits as observed in dairy and beef cattle and poultry. Hence such selection programs should have clear multi-disciplinary approach with the help of biologist, microbiologist, immunologist, epidemiologist, virologist, pathologist, environmental expert and the specialist in production systems management.

Recent developments in bioinformatics and the reported success in marker assisted selection could pave the way for new generation selection programs in aquatic animal breeding programs. Though the reports on selection for disease resistance in aquatic animals is limited, efforts are on for selective breeding of salmon against furunculosis, infectious pancreatic necrosis (IPN) and Infectious salmon anaemia (ISA) [19, 20] and the selective breeding of penaeid shrimp for Taura Syndrome Virus (TSV) [21].

Conclusion: Owing to the typical nature of aquaculture, prevention of diseases is going to be the primary objective in the coming years. Research and development in the country needs to be focused mainly to devise strategies for prevention of diseases through development of probiotics, vaccines, immunostimulants, genetic selection programs and through environmental interventions targeting the economically important species of aquatic animals cultured in this region.

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Quality control of biological products

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Introduction

Biological products, also called biologics are substances that include microorganisms or their products, sera, toxins, or analogous products of natural or synthetic origin that are intended for animal/human use for the purpose of treatment. These act primarily through the direct activation, supplementation, or modulation of the immune system or immune response. Such products include, but are not limited to, vaccines, bacterins, allergens, antibodies, diagnostics, antitoxins, toxoids, immunostimulants, antigenic or immunizing components of microorganisms to prevent, diagnose and treat or cure human / animal diseases.

Examples of biological products include:

- Vaccines

- Toxoids/bacterins

- Immune sera (anti-toxin & anti-venom)

- Immunostimulants

- Blood and blood products (e.g., platelet concentrate, plasma, albumin)

- Allergenic extracts

- Gene & cellular therapies

- Immunomodulators

- *In vivo* diagnostics (e.g., Tuberculin PPD)

Overall control on the quality of biological products during the manufacturing process is essential to ensure that the consumers received biological product of high quality. It ensures the biologics are pure, safe, potent, and effective to protect the health and enhance the well-being. In this article, we focus on vaccines, which are important biological products for the prevention of diseases. Vaccines are prepared in various forms- live attenuated (naturally occurring avirulent viruses as well as viruses modified by passage in different animal species or cell culture), inactivated and the recombinant

genetically engineered vaccines. Both live and inactivated vaccines may be formulated with adjuvants to enhance their efficacy. In principle, the antigen production process is the same for live and inactivated vaccines.

The quality of vaccines depends on the quality of the starting materials, manufacturing process, building/ facilities, equipment and most importantly, the personnel involved. All vaccines should be manufactured under carefully controlled and monitored conditions to ensure a uniform and consistent product of high quality. The implementation of good manufacturing practices (GMP) guidelines and quality assurance (QA) in manufacturing facility will ensure the quality of products in the market that will protect the public against the risk of buying and using substandard products.

Importance of QA and GMP-compliance in vaccine manufacturing

The quality, safety and efficacy of any biological product is the primary responsibility of the manufacturer. However, the regulatory authorities have to establish the standards and procedures to ensure that the products reach the required standard. In recent times, regulatory authorities insist on QA and GMP-compliance in the manufacturing of biological products. They are of particular importance in the manufacture of veterinary vaccines since such products have the following specific characteristics:

- The active ingredients of the vaccines are almost always produced by the manufacturer unlike the chemicals which are produced by another industry.

- Vaccine production usually involves cultivation of appropriate organism and the use of substances of animal origin, which makes it easy to introduce a contaminant and amplify low levels of contamination.

- As the end product does not go through a final sterilization step prior to final formulation, its constituents should be particularly well protected against contamination and cross contamination.

The biological products are manufactured using biological processes which inherently are variable due to variability in cultivation of cells or extraction of materials from living organisms. This necessitates strict adherence to GMP for all steps from obtaining active ingredients to packing of finished products.

General principles of good laboratory practices (GLP)

A biological product must be pure, safe, potent and efficacious. To produce such a product there must be a proper facility that has been inspected and approved as appropriate for the manufacture of safe biological products. These laboratories also perform activities such as distribution of the products. Therefore, close attention must be given to all the

parameters associated with the production and distribution of biological products, to ensure a good-quality product production in accordance with good laboratory practices (GLPs).

GLPs are a set of rules, operating procedures and practices that describe how laboratory procedures, tests and studies are planned, performed, monitored, recorded and reported, to ensure the quality and integrity of the data generated by a laboratory. These procedures and practices should be detailed and written in a clear manner, so that someone unfamiliar with them could perform the work, such as, for example, the operation and/or calibration of an instrument, the speed at which to run the centrifuge and the way in which to handle record charts, etc. These procedures and practices are generally written by the manufacturer and approved by the regulatory authority.

Some of the important aspects of GLPs are listed below.

Management:

Laboratory management is responsible for providing appropriate facilities, qualified personnel and good equipment, reagents and materials and for maintaining personnel records and ensuring that written and approved standard operating procedures, protocols and schedules are established and documented for all aspects of production. The laboratory should include a person responsible for ensuring that GLPs are in place, a technical manager who has the overall responsibility for production and a quality control manager who is responsible for all aspects affecting product quality, including maintenance, calibration, validation, monitoring of equipment and instruments and testing of the final product.

Personnel:

Before working with a particular organism or agent, management must evaluate the potential for human infection and, if needed, implement immunization and / or other necessary precautions. Production personnel must be competent in microbiological and good laboratory techniques through education and / or training. Before entering a biological production area, personnel should either change their clothes for clean laboratory clothing or cover their street clothes with appropriate laboratory garments. Hair covering, face masks, gowns and shoe covers should be used in production areas. Eating, smoking or any unsanitary practice should be prohibited in the production area. To maintain a high level of competence, staff should receive periodic training in laboratory techniques and quality control procedures.

Sterilization:

There should be written standard operating procedures (SOPs) for the washing and sterilization of all containers, instruments and equipment parts that would come in contact with the product. Proper recording of each sterilization cycle should be made to ensure that the proper time, temperature and/or concentration of the sterilizing agent have been achieved. Items subjected to sterilization procedures should be labelled and dated.

Labelling:

Labels for identification should be placed on all ingredients, components of a biological product, biologicals in any stage of preparation and completed biological products. The label should include the date of preparation and the initials of the preparer.

Management of seed organism:

Data concerning the master seed strain will be required to be submitted to the licensing authority. Therefore the laboratory must possess the following information on the seed organism:

- Origin and history of preparation

- Physical, chemical and biological properties, including antigenicity

- Growth ability

- Level of attenuation; marker and stability of attenuation

- Shedding of the agent from inoculated target animal

- Evidence of contact transmission

- Evidence of freedom from extraneous agents including viral, bacterial, mycotic and chlamydial organisms (periodic assays to be scheduled)

- Evidence of increasing virulence on serial passage in experimental host systems

- Results of tests by the manufacturer on three batches of trial products produced using standard manufacturing techniques.

Responsibility for the storage of organisms to be used as master seeds should be assigned to a particular individual. All vials of seed material should be labelled and stored in a secure location and the record for all seeds should contain a documented history, test results and an accurate inventory. Protocols for the testing procedures used should also be on file.

Outline of production:

Detailed outline of production containing a protocol and guidelines should be available for each biological product. Outline should comprise the following sections.

Composition of the product:

It includes the source and passage history of the organism(s) and, if applicable, the relative proportions of organisms in the product.

Cultures:

- Protocols and schedules (or frequencies) for identifying the organism(s) and frequency of identification;

- A protocol for determining the purity of culture(s) and, if applicable, the virulence of the organism(s) as well as the range of passage levels or subcultures to be used in production;

- The composition of the media to be used for seed and production cultures; sources of media ingredients, eggs, cell culture or tissues used; protocols for production of the media; and the methods used to determine the growth promoting qualities of the media and their freedom from contaminants.

- The protocols for production of media should include the formula, source and quality of ingredients; instructions on the storage and handling of ingredients; the quality of water required; equipment; the quality of glassware; procedures for product formulation and testing; the conditions for storage and handling of formulated media; and the product expiry date.

- The protocol for testing the sterility and growth-promoting qualities of media should include preparation and testing of QC media, the source and care of QC cultures and media performance testing. Records must be made concurrently with the performance of successive steps in the production and testing of each lot of medium.

- The record for each lot of medium should contain the name of the supplier, the lot number, the date of purchase, the date the seal was broken for each medium ingredient; the pH and osmolarity of the medium; the date the medium was prepared; and the initials of the preparer.

The outline of production should also include:

- A description of the containers used to grow organism(s) and instructions on how they are to be sterilized.

- Storage conditions for seed cultures.

- The protocol for preparing inoculum.

- The technique for inoculation and the titre and volume of the inoculum for each size and type of culture container.

- The duration and conditions of incubation.

- A description of the characteristics of growth and the characteristics of contamination.

Harvest:

- The minimum and maximum time allowed between inoculation and harvesting and the characteristics of the culture at harvest.

- The protocol for the preparation and handling of cultures for harvesting.

- The protocol for harvesting.

- Criteria for acceptable harvested material and the procedure for determining acceptability.

- Instructions for the handling of discarded material not used in production.

- Any additional pertinent information.

Preparation of the final product:

A detailed description should be given of every step from the harvest of the antigenic material to the completion of the product in the final container, emphasizing the following:

- The method of inactivation, attenuation or detoxification, if applicable.

- The composition of the preservative, adjuvant or stabilizer, the stage of production and the method of addition.

- The protocol for the method and the degree of concentration;

- If the product is standardized to a specific concentration of antigen, the procedures used to achieve this concentration and the calculations made in doing so should be given.

- The volume of fill for each size of vial.

- A description of the method and technique for filling and sealing the final container.

- The protocol for lyophilization including procedures for determining the moisture content.

- The amount of antigenic material per dose or doses in the final container and how this is determined.

- Conditions for storage of the finished product.

Testing:

A description should be given of how samples of the final product are collected, stored and tested. Protocols should be provided for the determination of purity, safety, potency, moisture content and any other test performed on the product. Each test protocol should include the minimum requirement for a satisfactory test.

Labelling:

All biological products should be properly labelled. The final container label should include the following information.

The name of the product; the name and address of the producer; the recommended storage temperature; the number of doses in the vial; the use, dosage and route of administration for each animal species for which the biological product is recommended; the expiry date; the serial number; and warnings or cautions, if applicable. The expiry date is based on the earliest date of harvest and the date of the last satisfactory potency test. If applicable, the date of lyophilization should be given. A stability record should be established by testing each serial for potency at release and at the approximate expiry date. The label should also contain warnings or cautions for the products, if any.

Storage:

Completed biological products should be stored at 2° to 8°C, unless a different temperature has been shown to give better stability.

Records:

Each biological production facility should keep detailed records of all the activities carried out within the establishment. These should include a daily log of production area use. Records should be made concurrently with the performance of successive steps in the production of each lot and should contain the date and time of all critical steps, the identity and quantity of all ingredients added or removed and sufficient detail to give a clear understanding of each step in the preparation of the product. The charts and temperature records made during preparation of ingredients, sterilization of equipment or manufacture

of a product are part of the record for the lot being produced. For each lot there should also be detailed records of the tests performed on ingredients, seeds, the product during manufacture and the completed serials or sub-serials of the product. The facility should have a record of the location of all biologicals being prepared and the quantities held in storage and distribution channels.

Quality control of vaccines:

Quality control tests employed in vaccines is illustrated with the example of inactivated Foot-and-mouth disease vaccine.

Pre-production quality control:

Seed virus management is an important aspect of pre-production quality control. Seed virus management involves the following aspects:

Characteristics of the seed virus: Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture (both in monolayer and suspension culture), virus yield, stability and broad antigenic spectrum. MSVs should be selected in accordance with the epidemiological importance of each variant.

Method of culture: New vaccine strains are derived through the establishment of MSVs from local field isolates by adapting them to growth in suspension or monolayer cells by serial passage. In order to remove the risk of contaminating lipid-enveloped viruses, it is recommended that putative MSVs undergo a validated organic solvent treatment prior to, or during, adaptation. It is preferable to keep the number of passages in cell culture to a minimum as there is evidence of antigenic 'drift' of FMDV during this procedure.

Validation as a vaccine: The criteria that are considered to validate a virus strain as vaccine are, i) MSVs must be antigenically and, if possible genetically, characterised and proven to be pure and free from all extraneous agents listed by the appropriate licensing authorities. ii) Homology should be established with the original candidate isolates and effectiveness against the circulating strains from which they were developed should be proven.

In-process quality control:

- *Virus titer:* In general, virus titres reach optimum levels within about 24 hours of the cell culture being infected. The time chosen to harvest the culture depends on serotype of virus. Extent of cell death (cytopathic effect) is the main criteria to decide the time of harvest

- *Virus concentration:* may be assessed by an infectivity test, sucrose density gradient or serological techniques. It is preferable to use a method for measuring antigenic mass, such as sucrose density gradient analysis, as well as one that measures infectivity, as the two properties do not necessarily coincide and the different methods may complement one another.

- *Inactivation of virus:* During inactivation of the virus, timed samples should be taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of cell cultures proven to be highly susceptible to FMDV, e.g. BHK or bovine thyroid cells. The $\log_{10}$ infectivities of the timed samples are plotted against time, and the inactivation procedure is not considered to be satisfactory unless at least the latter part of the slope of the line is linear and extrapolation indicates that there would be less than one infectious particle per 10,000 litres of liquid preparation at the end of the inactivation period.

Post-process quality control (tests on the final product):

- *Safety:* Tests for innocuity (non-infectivity) are most effectively carried out on the bulk, concentrated, inactivated viral harvest. For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in an in-vivo test in an appropriate number of cattle.

- *Potency:* Vaccine potency is estimated in vaccinated animals either directly, by evaluating their resistance to live virus challenge, or indirectly, by inference from the levels of specific antibody induced by vaccination.

- *Duration of immunity:* Wherever possible, vaccine manufacturers should demonstrate the duration of immunity for their specific formulation in each species for which it is indicated.

- *Stability:* The stability of all vaccines, particularly oil emulsion vaccines, should be demonstrated as part of the shelf-life determination studies for authorisation.

Batch control:

- Sterility: The bulk inactivated antigen, the adjuvants, the dilution buffers and the final formulated product should undergo sterility testing as prescribed in Indian Pharmacopoeia. This may be carried out directly with components of the vaccine and the final product, but the preferred method is to collect any contaminating microorganisms by membrane filtration of the material to be examined and to

detect any organisms present by incubation of the membranes with culture media. The latter procedure allows the removal of preservatives, etc., which may inhibit the detection of microorganisms.

Sterility test is commonly performed using Fluid Thioglycollate Medium (FTM) for identification of aerobic and anaerobic bacteria and Soyabean-Casein Digest Medium (SCDM) for detection of fungal and aerobic bacterial contamination.

- *Innocuity:* The test for innocuity is an in-process test that should be carried out for every batch of antigen. Following inactivation, a sample of each batch of inactivated antigen representing at least 200 doses should be tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures, preferably of the same origin as those used for the production of antigen.

- *Safety:* This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions. For the purposes of batch release, each of at least two healthy seronegative cattle is inoculated by the recommended route of administration with double the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Should any of the animals develop clinical signs of FMD, the vaccine fails the safety test.

- *Potency:* Potency is only examined on the final formulated product. Antigen load can be used as an indirect indicator of potency, provided that (i) Good Manufacturing Practice ensures that the method of manufacture and formulation of the antigen/vaccine remains the same, (ii) a correlation has previously been established between antigen load, serological response and protection against challenge, and (iii) a suitable alternative test measuring the serological response to immunisation has been carried out with satisfactory results.

Usually, materials of in-process and final products will be retained in sufficient quantity with appropriate storage for repetition or confirmation of batch control.

Importantly, record keeping of all steps in manufacture and testing for at least two years after the expiry date of the product is necessary and it should be available for inspection by drug/biological control authority.

Three Rs Approach in the Quality Control of Vaccines:

Under the standard protocol, there is a spectrum of evaluation steps including characterization of seed material and ingredients, and laboratory- and host-animal safety and efficacy studies. Also, post-production tests include batch tests for purity, safety,

and potency. For the production and testing of regulated products, animals are used to validate product requirements for safety, potency, and efficacy. In this context, there is a rising demand to incorporate alternative methods to reduce, refine, and replace the use of animals in the development and testing of biologics. Although there has been considerable progress in the application of the 3Rs principle, there is still a need to ensure that correlation to protection is still demonstrated by the in vitro methods. Research and development incentives are also needed to address knowledge gaps and accelerate the development of new and alternative methods of international acceptance.

Summary

The quality of biologicals used to diagnose, prevent and cure diseases is a major component of strategies to improve animal health in the country. A reliable supply of pure, safe, potent, and effective vaccines is essential for maintenance of animal health and the successful operation of animal health programmes. The approach to ensuring quality of the biologics vary from country to country depending on regional requirements. However, proper standards and production controls are essential to ensure the availability of consistent, high quality products for use in animal health programmes. Quality assurance covers all matters that collectively influence the quality of a product. It is the sum of the organized events ranging from process control, improvement and inspection, testing of the quality, efficacy and safety of the vaccines to assurance achieved through competent authority procedures. It is expected that the end user receives the products that are safe and effective, apart from being produced under appropriate standards of good manufacturing practice. Therefore, it is essential that the highest standards are maintained in the production and supply of biologicals.

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Genetic Selection For Disease Resistance

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Selection

Genetic improvement programmes are being operated for various aquaculture species world-wide which typically involve periodic evaluation, selection and culling in a population of fish. Selection is the process of choosing from a population, individuals to be used as parents for producing the next generation of progeny and is a crucial aspect of the genetic improvement program because the selected fish would decide the genetic make-up of the progeny and hence their performance. All other non-selected fish are culled from the population to prevent them from breeding and producing progeny. The major goal of selection is to improve one or more desirable traits in the population of a species.

Disease resistance

Disease resistance has been a target trait for improvement in most of the genetic improvement programs as the overall health of the fish population is linked to economics of fish culture production. Disease resistance (DR) is a threshold trait where the expressed phenotype is qualitative (a fish is identified as healthy or infected) and the underlying genetic control is quantitative (influenced by the number of genes). Most of the time, the word tolerance is used synonymously, which is little different from resistance. Resistance is defined as the ability of the host to block pathogen replication and tolerance is the ability of the host to limit the impact of infection [1].

Recording of disease resistance

Any measurable trait could be taken up as a selection criterion for improvement in a selection program. However, the DR is a very difficult trait to measure because at any time, fish at different stages of infection exist and one cannot clearly differentiate a healthy fish from an infected one. Considering DR as a resistance trait against a single disease is apt for fish selection programs. DR was included as a target trait for improvement in Salmon breeding programs way back in the 1990s. Mostly the DR is measured as a categorical trait like survival/mortality of fish after infection or as a continuous variable like time taken to the death of fish following infection. Accordingly, different statistical approaches are to be employed depending on the type of data recorded. The data type considered should be simulating the natural infection so that the selection based on recorded data would give dividends over generations of selection. Ideally, the data recorded during natural disease outbreaks could be analysed to understand the genetic

component of resistance to a disease of fish. As prevention of disease is given more thrust during fish culture operations, obtaining mortality data from disease outbreaks is difficult. Any statistical procedures applied to understand the genetics of disease resistance require pedigree data which is challenging to obtain for field samples though costlier alternatives using molecular markers exist to overcome this challenge. Another problem with field data is to pin-point the cause of mortalities during natural infections as multiple factors could have contributed to the precipitation of disease outbreak. Therefore, planned challenge experiments in controlled conditions are generally conducted to generate survival data and understand the genetics of disease resistance. In controlled experiments, as the pathogen at lethal doses are introduced into the fish either orally or intramuscularly or through immersion or anal routes, the cause of death could safely be attributed to the pathogen. Stronger correlations should exist between field data and experimental data so that the latter data could be used for fish selection. For example, in case of furunculosis disease in Atlantic salmon, high genetic correlations were reported between field and experimental data [2].

Basis of selection

In a selection program, to improve DR trait in the selected fish population, the trait has to be recorded on the fish candidates. Thereafter, the trait is evaluated using suitable models to obtain estimated breeding values (EBVs) which form the basis of selection. In aquaculture species, family selection is practiced, as the fecundity is high compared to terrestrial farm animals. The EBVs are obtained for a family of fish and either a family is retained or culled from the program. In some of the programs, family selection is followed by within-family selection to select a few better fish from each family. Either the survival data is directly used for selection or other traits like haemocyte count, that is indicative of health, could be used.

Individual selection is not preferred for DR because neither the fish that participate in challenge experiments nor the fish that survive in field outbreaks can be used in breeding program as they might pose a risk of disease introduction. This risk forces breeders to practice family selection on the sibs of fish that underwent challenge tests. Selection on the basis of relatives like sibs, slow down genetic gains as the selection based on individual performance has more accuracy than the one based on relatives.

Strategies to understand genetics of disease resistance

Recent technological developments resulted in expansion of public genome resources like genome sequences, EST datasets, and genotyping assays etc. for several fish species. Genome sequences have been generated for commercial species like rainbow trout [3], Asian seabass [4] etc. High throughput SNP genotyping arrays were developed

for species like Atlantic salmon [5] and rainbow trout [6]. Vast genomic resources are being exploited by researchers to find the genetic factors contributing to disease resistance in fish. There are mainly three strategies followed: candidate gene approach, Quantitative Trait Loci (QTL) mapping and gene expression studies.

The Candidate gene (CG) approach assumes that variations in certain genes involved in certain biochemical pathways might be responsible for variation in the phenotype of the trait under consideration. The pre-requisites to use CG approach include knowledge about biochemical pathways and sequence information for the gene candidates.

The QTL mapping approach involves large scale genotyping of DNA markers and then associating the marker genotypes with recorded phenotypes. Any number of markers could be tested for their association with phenotype. Once validated, the genotype data at certain loci could form the basis for the selection of fish. Such a selection process based on markers is called Marker Assisted Selection (MAS). Synonyms for MAS in literature include 'marker assisted breeding' and 'marker aided selection'.

The expression pattern of genes would help scientists to unravel specific genes that play a crucial role in regulation of a trait. Approaches like microarray require knowledge on sequences of genes under study whereas the transcriptomics approach does not require prior knowledge of sequence data. The differentially expressed genes in fish of extreme phenotypes could be further studied to document the genetic control of the trait.

Two case studies where attempts were made to improve the resistance to specific diseases are discussed below. One involves the Taura Syndrome Virus (TSV) in shrimp where the resistance to this virus is heritable and could be improved by conventional selection. The other is about White Spot Syndrome Virus (WSSV) where the resistance trait has very low heritability and cannot be improved by conventional selection schemes.

Taura syndrome virus (TSV) disease

The TSV had a huge economic impact on the shrimp farming industry during the 1990s when it exhibited as an epizootic disease in *Litopenaeus vannamei* first in Ecuador and later in America and Asia. This deadly virus infection used to result in cumulative shrimp mortalities of up to 80-90% in infected ponds [7]. Today, though TSV is listed in OIE for important diseases affecting shrimp, it is no more considered a deadly pathogen, thanks to the selection programs that improved shrimp resistance to TSV. The U.S. Marine Shrimp Farming Program (USMSFP) had initiated a selective breeding program in 1995 for TSV resistance in *L. vannamei*. The selection program included challenge tests and sib-selection. One or two cohorts of about 50-80 families of shrimp were orally challenged each year with TSV virus to record survival data [8]. Thereafter, the sibs of the families performing best in challenge trials that had never been exposed to the pathogen

were selected for producing the next generation. The cycle of oral-challenge and sib-selection was repeated for generations. After one generation of selection, the selected families showed 18.4% increase in survival post-TSV challenge compared to non-selected families [9]. After 15 generations of selection, the survival per cohort increased to more than 80%. The challenge tests were conducted with several isolates of TSV including the most dreadful isolate of Belize. A few families in each cohort also recorded 100% survival after viral challenge. After making significant gains for TSV resistance, selection efforts were shifted to improve growth and grow-out survivals. However, similar family-based selection efforts towards improving resistance to other pathogens like WSSV and yellow head virus (YHV) did not yield significant results.

White spot syndrome disease

The white spot disease is caused by WSSV, which is considered the most important pathogen for shrimp. The virus is capable of inflicting 100% mortality in shrimp ponds within a span of 7-10 days. The rapid mortalities were also due to secondary infections resulting from the cannibalistic nature of the species. Researchers have attempted to improve resistance to WSSV in *L. vannamei* where genetic improvement programs are being operated. However, either low heritabilities were obtained or very limited evidence was found for genetic variation for WSSV resistance in shrimp [10, 11]. The WSSV resistance is also reported to be negatively correlated with growth rate [12]. Considering these bottlenecks, the ICAR-Central Institute of Brackishwater Aquaculture, Chennai in collaboration with NOFIMA, Norway started a collaboration program in 2008 to develop genome resources that would lead to practising MAS for WSSV resistance in tiger shrimp. First, utilizing the genotype data obtained on an Illumina iSelect genotyping array for cSNPs derived from transcriptome data, a dense linkage map has been developed [13]. The map contains about 3959 SNPs in 44 linkage groups. Later the genotype data of WSSV-challenged shrimp was used for association studies with survival data. The study documented a few QTL regions for WSSV resistance in tiger shrimp [14]. Fine mapping of these QTL regions may finally pinpoint the causative loci responsible for genetic variation in WSSV resistance.

To conclude, disease resistance is a threshold trait and is extremely difficult to record. Either survival data or some indirect parameters indicative of resistance could be used as traits for improvement in selection programs. Many a times, improvements in DR could not be made through selection programs as the trait may be negatively correlated with important primary traits under selection. Good genetics can never overcome bad management or environment. The improvements made in disease resistance cannot be used as a shield against poor disease-control measures at field. Farmers should always try to prevent the pathogen entry into the system by adopting the best possible management practices.

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Environmental stress and its prevention in aquatic animals

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Stress is generally defined as a condition in which the dynamic equilibrium/homeostasis of an organism is disturbed as a result of intrinsic or extrinsic stimuli. Bayne [1] defined stress for aquaculture as "a measurable alteration of a physiological steady-state that is induced by an environmental change and that renders the individual more vulnerable to further environmental change". Normal physiological balance disturbed by anything external or internal can be reflected to be stress. Stress has two forms: one benevolent (shapes evolutionary progress and strengthens a species ability to survive), and the other malevolent (weakens normal physiological processes of host to pathogens). In addition to extrinsic environmental factors, intrinsic factors like sex, size, moulting stages and nutritional status influence the ability of animal to invading pathogen. Symptoms of stress are lethargy, absence of feed intake, slow growth, moulting problems and hyperactivity, which leads to the incidence of diseases in finfish and shrimp culture [2, 3].

Recent research on stress response studies has identified the levels of glucose, lactate and osmoregulatory capacity as tangible markers for measuring the stress response in crustacean species. Additionally, other metabolic variables like hemocyanin, total proteins, total lipids, triglycerides, and cholesterol are also suggested to be of useful indicators to monitor the physiological condition of shrimp.

Brackishwater aquaculture under varying source waters

Brackishwater culture species, particularly shrimp *Penaeus monodon* and *P.vannamei,* and finfish *Lates calcarifer,* are being cultured by farmers in sea, brackish and fresh waters. High salinity and clear water with less plankton always cause shrimp stunt, but high salinity water affects shrimp only at the juvenile stage when they mainly consume zooplankton. Bacterial infection and pond bottom deterioration, generally caused by over blooming of phytoplankton as in brackishwater ponds, are not observed in seawater-based culture ponds. Culture in freshwater requires a closed system to avoid viral diseases as virus carriers grow very fast in fresh water. Groundwater may differ significantly in terms of its relative ionic composition compared to seawater. Most saline groundwater is deficient in potassium although other key ions such as sodium, chloride, calcium and magnesium can also vary considerably depending on the aquifer. Major ion deficiencies can have serious physiological consequences ranging from stunted or poor growth to asphyxiation, oedema and death. Potassium has an essential role in regulating sodium and hence there is a need to supplement potassium as and when required.

Environmental Stress

Survival, growth, metabolism and other physiological processes of shrimps are generally decided by environmental characteristics [4]. The maintenance of good water quality in ponds is essential in providing a low-stress rearing environment for aquatic animals. Animals under stress due to various environmental factors show higher levels of biogenic amines including noradrenaline and dopamine, which are immune suppressive in nature, increasing susceptibility to pathogen infections.

Causes of stress in aquaculture ponds

Many environmental factors will affect the immune response, leaving animals more susceptible to disease. Pond environmental parameters like temperature, salinity, dissolved oxygen, pH, ammonia, nitrite and hydrogen sulphide have greater impact on the immune functions of shrimp. Extreme ranges of these parameters have proven to have adverse effect on the cellular components of the shrimp immune system. The important water and soil stress parameters are:

i. *Improper pH levels*

Optimum water and soil pH for aquaculture are between 7.5 to 8.5 and 6.5 to 7.5, respectively. Acute stress occurs when change in pH is sudden and chronic stress occurs when pH continually rises or lowers. The change in pH affects the metabolism and physiological processes of aquatic animals. When pH is more than 9, the resultant alkaline environment is dangerous as percentage of un-ionized ammonia increases, which is more toxic (http://www.fao.org/docrep/field/003/ac210e/AC210E09.htm). Waters with low pH will cause an increase in the fraction of anionic sulphide (H_2S) as well as physiological disorders in shrimp.

ii. *Fluctuations in temperature*

The temperature below and above the optimum range (28 to 32°C) is known to weaken the immune status of the shrimp, making it more susceptible to diseases due to *Vibrio*. Aquatic animals reach a limit of physical and nutritional tolerance and stays stationary at the bottom when temperature is 33°C in poor quality water or 35°C in good quality water. Unequal distribution of temperature in the pond with high temperature at littoral zone and low temperature in benthic zone results in thermal stratification in deeper ponds. This degrades the water quality by accumulation of methane, hydrogen sulphide and ammonia. Use of aerators helps in breaking the thermal stratification. In general, the lower temperatures reduce rather than stop the viral replication [4, 5]. In brackishwater shallow ponds, where regular exchange between the tidal water and the pond water is not maintained during the hot dry months, the temperature of pond water may shoot up beyond the tolerance limit causing mortality of reared shrimps.

iii. Improper salinity

An optimal salinity range of 10 to 35 ppt is considered optimum for the growth and proper metabolic processes of brackishwater species though many can tolerate wider range of salinities. White shrimp can tolerate a salinity range of 2 to 40 ppt. Salinity fluctuation during the day should not exceed 5 ppt per day. Animals use the osmoregulatory mechanism to sustain the equilibrium and protect the internal tissues. At iso-osmotic salinity levels shrimp exhibit higher resistance against pathogen infection due to competent immune system. Interaction between temperature and salinity are, one modulating the effect of the other in the metabolic response [6].

iv. Low dissolved oxygen

The optimum DO level required by the aquatic animals for normal physiological and biochemical activities are more than 3mg/l. Water temperature, respiration and the level of organic matter are the factors which affect the amount of dissolved oxygen (http://www.fao.org/docrep/field/003/ac210e/AC210E09.htm).When temperature increases, the oxygen solubility in water decreases. Oxygen has direct effect on feed intake, metabolism and indirect effect on environment conditions which affect growth and production. In an intensive culture system, low DO levels (less than 2.8 mg/l) due to decomposition of accumulated feed and the animal faeces cause anoxic condition and it is known to influence the growth, survival, feeding, moulting, osmoregulatory capacity and immune response of Penaeid shrimps.

v. High metabolites concentration

It is the combinations of two or more metabolites (ammonia, nitrite, sulphide) that are responsible for retarded growth or mortality of shrimp in ponds. The concentration of total ammonia nitrogen (TAN) in intensive grow-out ponds increases as culture progresses and levels of more than 1.0 ppm are toxic. The percentage of the toxic un-ionized ammonia form increases as pH and temperature rise during the day and can reach critical levels. Shrimp growth and survival can be reduced with long-term exposure to un-ionized ammonia at 0.1 ppm and short-term exposure to as low as 0.4 ppm.

Nitrite concentrations in aquaculture ponds are usually very low (<0.1mg/l) and >0.5 mg/l is toxic. Nitrite can accumulate in haemolymph up to 10 fold higher than in water via active chloride uptake mechanism and passive entry and leads to reduced levels of oxyhaemocyanin and increased deoxyhemocyanin. The source of nitrite nitrogen is through the addition of feed, fertilizer and manure. Nitrite is more toxic in low saline conditions compared to brackish and seawater based culture ponds.

Un-ionized hydrogen sulphide is toxic to aquatic organisms. Concentration of 0.01 to 0.05 mg/l of h_2s may be lethal to aquatic organisms and any detectable concentration is undesirable. The presence of sulphide affects the immune parameters like total haemocyte count, phenol oxidase activity, phagocytic activity and clearance efficiency, thereby making the shrimp more susceptible to pathogenic infections like vibriosis. Hydrogen sulphide toxicity is inversely related to dissolve oxygen concentration. A typical feature of sludge accumulating in shrimp ponds is the black colour and a smell of hydrogen sulphide. Feed acceptance by shrimp was significantly lower in pond areas with sulphide containing sediment [7].

vi. Increase in organic load

Unutilized feed, carbonaceous matter, dissolved solids, dead plankton etc. settle at the pond bottom and results in the accumulation of organic loads. The change in the bottom in terms of increasing organic matter load should be monitored regularly for the management of the pond bottom. When an input of organic waste exceeds the supply of oxygen, anaerobic condition develops. This reducing condition can be measured by redox meter. Redox-potential is represented as E_h, which indicates whether the bottom soil is in reduced or oxidized condition. Reduced or anaerobic sediments may occur at the pond bottom of heavily stocked pond with heavy organic load and poor water circulation. Under anaerobic condition of the pond bottom, reduced substances such as H_2S, NH_3 which are toxic to benthic organisms are liberated and diffused into water phase.

vii. Overstocking

The growth and feed intake of cultured shrimp affected by increasing stocking density is a critical factor. An increase in the stocking density goes together with more organic load, high reducing conditions, low pH and dissolved oxygen, and these changes in water quality affect the growth and survival of shrimp. Metabolites (toxic to the animals) directly proportional to stocking density have been involved in inhibiting their growth [8, 9].

Management measures for prevention of stress

In view of the observed effects of environmental stress on the immune system of cultured shrimp, the management strategies should include maintaining the optimum condition of pond environmental parameters. Good pond management is critical as the water quality can deteriorate quickly due to the accumulation of organic matter from uneaten feed, faeces, dead shrimp and algal bloom crashes. Pond water quality is influenced by both environmental and management factors. Regular monitoring of water and bottom soil in culture ponds for pH, DO, ammonia, nitrite and H_2S is the key in protecting the losses due to diseases.

A. *Intake water treatment*

Polluted or self-polluted source water through aquaculture causes slow growth, disease outbreak and accelerated mortalities in shrimp. A reservoir has to be an integral component and should be attached to grow-out ponds for sedimentation to settle organic loads and silt and chlorination treatment. Adding treated water from the reservoir (approximately 30%) throughout the crop is essential to prevent excess salinity which may gradually increase through evaporation.

B. *Water exchange*

Traditionally, the management of water quality is through water exchange to reduce organic load and flush excess nutrients and plankton (cyanobacteria) out of the pond. However, due to increasing farm density, deteriorating intake water quality and rise in viral diseases, the use of water exchange as a method of pond water quality management is questionable. Minimisation of water exchange will prevent viruses and carriers/bacterial pathogens from entering the ponds and reduce the possibility of disease transmission into shrimp ponds. But the reduction of water exchange requires closer control of water quality parameters, effective sediment management, control feeding and reduction in stocking density. However, improperly managed closed system increases the risk of stressful rearing conditions, bad water quality and diseases in ponds. Hence, the best water management option is limited water exchange from treated reservoir to reduce the potential of disease introduction into the farms through intake water.

C. *Aeration*

In general, aeration to achieve more than 4 ppm of DO is related to production targets, stocking density, feed usage and salinity. Managing the concentration of DO in pond waters is very closely related to the amount and type of phytoplankton, the number and condition of the existing aerator, shrimp biomass, total organic matter content in the pond, and bacterial activity. Generally, one horsepower is suggested for 500 kg production. The placement of aerators is important to prevent localized deposition of sludge. Maintaining sufficient level of DO facilitates oxidation of ammonia to harmless nitrate by nitrifying bacteria.

D. *Feed management*

The practice of providing food for the shrimp is a trade-off between food source and water quality in the pond. It has been estimated that as much as 0.4 ppm ammonia can be added to the system for each 100 kg of feed used. Overfeeding, even in one feed, can lead

to sudden increases in ammonia, sometimes called ammonia spikes, a few hours later. The feeding quantity should be strictly controlled according to the weather, water quality, shrimp density and the actual flexibility to adjust food intake.

E. *Pond bottom management*

Pond bottom management is very important because most of the shrimp activities are performed in the pond bottom. Keeping the pond bottom clean will indirectly protect water quality and shrimp health. Ponds with soft sludge give poorer yields. However, earthen pond bottoms can be improved with oxygenation by the tilling of the pond bottom and followed by sufficient drying [10]. Water circulation by water exchange, wind or aeration helps to move water across mud surface and prevent the development of reduced condition. A central drainage canal in the pond may also help in the removal of organic waste periodically. Negative (-) redox value shows reducing condition, whereas positive (+) value shows aerobic condition of the pond bottom mud [11]. E_h of pond mud should not exceed -200 mV.

F. *Use of chemicals, disinfectants and probiotics*

Various chemicals have been recommended for reducing the load of harmful bacteria in the pond. There is very little evidence for the efficiency of these compounds. Most of the recommended substances are broad-spectrum disinfectants including quaternary ammonium compounds (Benzalkonium chloride), buffered iodophores and calcium hypochlorite. Effective use of scientifically proven products helps maintain optimum pond environment [12] and also to maintain the discharge water parameters from the cultured farms within the prescribed standards [13].

Conclusion

Two-pronged approach of combining pond environment management and health monitoring is the key for successful shrimp production. It is important to know how much shrimp can be supported by the pond environment (carrying capacity of pond). Although the ideal carrying capacity can be low, higher production volumes can be achieved by partial harvesting. The promotion of growth of natural planktonic or benthic microbial and microalgae communities (bioflocs and periphyton, respectively) present in the pond environment helps in the utilization of nutrients through autotrophic and heterotrophic processes accelerating the removal of organic and inorganic wastes, thus improving water quality. Regular monitoring of environmental parameters and timely mitigation using appropriate biological agents is the key to protect potential losses due to stress and opportunistic bacterial infections. Understanding the ecological processes occurring in shrimp culture ponds through regular monitoring will help to prevent stress to the animals.

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Probiotics for aquatic animals

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Introduction

The aquaculture industry is caught between demand for culture intensification on one side and economic viability and environmental sustainability on the other. The economic sustainability of the industry is heavily dependant on the health and welfare of the culture species of animals. Stress and diseases are considered as major impediments in the development of the aquaculture industry. Diseases in aquaculture are traditionally controlled using chemotherapeutic agents like antibiotics, anti-parasitic agents, antivirals, antifungal substances, etc. With increasing public awareness about the residues and development of drug resistance, it has been advised to search for alternative approaches. Application of natural products of animal and plant origin and beneficial microbes are some of the methods which can be investigated to help the host enhance its immune responses. Immunostimulants, vaccines and probiotics are some of the suggested concepts to follow prophylactic measures in aquaculture. Due to the abundance of information, ease of application and cost effectiveness, probiotics applications have been the most preferred disease preventive methods in aquaculture. Additionally, probiotics improve the level of host health, nutritional efficiency and the quality of culture environment. In natural conditions host microbiome in the body system helps the host in several metabolic functions including immunity and environmental microbiome help in bioremediation by mitigating the agricultural and industrial pollutants.

Though vaccines can be self-replicating, they are very specific against a particular pathogen and cannot be used for nonspecific stimulation of the immune system. On the other hand, immunostimulants, though have non-specific wide spectrum immune stimulation, lack self-replication and hence need to be applied repeatedly which may not be a practically or economically viable option for aquatic systems. However, the probiotics, being self-replicating, improve the general health, nutritional efficiency and environmental quality and thereby have an advantage over vaccines and immunostimulants especially in aquaculture practice.

Though our knowledge about probiotics has emerged from the observed mechanism in terrestrial animals, special attention needs to be given when they are discussed in the context of aquaculture. The effect of probiotic application in terrestrial animals and humans is limited to the digestive tract, whereas the effects extend to improved productivity, nutrition, disease resistance, water quality and the quality of the

effluent from the cultured ponds. With respect to aquaculture, probiotics can be classified into gut, water and soil acting and their combinations. Reports on the effects of probiotics on the immune system, digestibility, survival and growth in aquatic animals like fish, shrimp, crabs and molluscs have been published extensively during the last two decades. Similarly, the cost benefit analyses of probiotic application in growout shrimp farms also have suggested the beneficial effects through several field studies.

Definitions

Several researchers have studied the different mechanism of probiotic action and proposed the definition based on their observed effects. Hence several definitions with contrasting opinion have been suggested. However, the term probiotic comes from Greek words *pro* and *bios* meaning "prolife". The most widely quoted definition of probiotic as proposed by Fuller [1] was "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". This definition is suitable for gut probiotics considering only one of the several mechanisms of probiotic function. FAO reports [2] define probiotic as 'living microorganisms, which when administered in adequate amounts confer health benefits on the host'. Though inappropriate, Lazado and Caipang [3] defined probiotics as "live or dead, or even a component of the microorganism that act under different modes of action in conferring beneficial effects to the host or to its environment". However, an appropriate definition of probiotics from the aquaculture point of view was suggested by Verschuere and others [4] which encompass almost all the activities and beneficial effects. According to this, probiotic is defined as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment." Though several researchers have proposed different definitions, the original idea was from Nobel laureate Elie Metchnikoff who introduced the concept of probiotis to the scientific community in his book "The Prolongation of Life" published in 1907. He suggested that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" and linked the longevity of Bulgarians with the consumption of fermented milk products containing viable Lactobacilli.

Mode of Application: In hatcheries probiotics can be applied directly into the rearing waters, through live feed like *artemia* and rotifer or by immersion treatment [5]. Generally, live feeds used in hatcheries were suspected as carriers of pathogens affecting the growth and survival of fish larvae. Applications of probiotics in live feed cultures have shown to reduce the multiplication of pathogenic bacteria affecting the larval growth and

survival. Limited studies have also suggested the beneficial effects of probiotics applied as microbial matured water in fish larval rearing systems.

Probiotics in larval culture

The Success of fish hatchery depends almost entirely on the percentage of larval survival. High fluctuations in the rate of survival at each stage of larval development are a major constraint in the economic viability of the operation. Bacterial infections are considered responsible for such variations and live feeds, such as Artemia nauplii, are considered vectors for introducing pathogenic microbes into the larval rearing systems. Probiotics applied in algal cultures decompose organic matter and produce secondary metabolites making it a source of nutrition to fish larvae. Due to continuous flow systems in fish hatcheries, the establishment of probiotic bacteria culture environment needs regular application in large quantities. Hence introduction of probiotics through live feed, which requires less quantities and also possibly reduces the pathogenic microbial loads, is considered a better option.

Establishment of microbes in a system

To be effective, probiotic bacteria needs to be established in the culture environment both within and outside the host. The factors which determine the probability of microbial establishment in a new environment are salinity, temperature, oxygen concentration and quantity and quality of the feed. During the pond preparation use of drying and chlorination effectively removes the natural microbial loads in the aquatic system and application of probiotic favours the chance of microbes in the product to get colonized. In case of already established microbial population in the system, the regular application of exogenous consortia would increase their chance of establishment in a new environment. The establishment of exogenously supplemented microbe in the fish gut mucosa has been confirmed by several studies.

Mechanism of action

The beneficial effects of probiotics are attributed to one or a combination of mechanisms like inhibition of pathogens by the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, enhancement of the immune response, improvement of water quality, interaction with phytoplankton, production of digestive enzymes and/or source of macro nutrients. Each species in probiotic might induce multiple effects and a product with multistrain/multispecies of bacteria is expected to have synergy with complementary modes of action to enhance beneficial effects [6].

Effect of probiotics on gut mucosa

Regular administration of probiotics through the oral route has shown to alter the diversity and metabolic functions of gut microbiome. Further, probiotic microbes modulate the gut mucosal immunity, stimulate epithelial cell proliferation and differentiation and fortify the intestinal barrier in addition to competing with pathogens for binding sites [7, 8]. Further, probiotics regulate the gut-brain axis through improved secretion of neuroactive and neuroendocrine molecules by gut microbome leading to beneficial effects in neurological disorders [9]. Regular use of probiotics in fish larval culture system has shown the establishment of these microbes on egg surface and in gut leading to significant improvement in the larval survival. Similar observations in adult fish also suggest that beneficial microbes in the probiotic product establish and produce some antibacterial compounds which inhibit the opportunistic pathogens. Since the duration of establishment depends on the ability of probiotic bacteria to survive the host environment, regular application is recommended.

Immune stimulating effect of probiotics

Effective functioning of immune system has been the hallmark of health in all the living beings on the planet including aquatic organisms. Compared to other beneficial properties on the immune stimulating effect of probiotics which have been extensively reported in human, veterinary and aquatic medicine suggest the term "immunoprobiotics" to identify those classes of bacteria [10]. Immunological parameters suggestive of the immune stimulating effect of probiotics have been reported through improved activity of lysozyme, phenoloxidase, peroxidase, anti-protease activities, phagocytosis and respiratory burst activity.

The molecular mechanism behind the immune stimulatory activity of probiotic was attributed to the components of bacterial cell wall envelope, S-layer proteins, capsules and pellicle. The pattern recognition receptors (PRRs) present in the immune system recognize the microbe associated molecular patterns (MAMPs) like, lipopolysaccharides (LPS), peptidoglycan, flagellin and microbial nucleic acids. The most prominent 11 types of PRRs identified in shrimp are b-1,3-glucanase-related proteins, b-1,3-glucan-binding proteins, C-type lectins, scavenger receptors, galectins, fibrinogen-related proteins, thioester containing protein, down syndrome cell adhesion molecule, serine protease homologs, trans-activation response RNA-binding protein and Toll like receptors [11]. Similarly, four types of PRRs reported in fish are toll-like receptors (TLR), NOD-like receptors (NLR), C type lectin receptors (CLRs), and peptidoglycan recognition proteins

[12]. Regular administration of probiotic products has shown to increase the expression of immune regulatory genes which was confirmed by comparative transcriptomics [13]. Since the immune system modulating effects of probiotic is strain and host-dependent it is not appropriate to extrapolate the observations across the bacterial species and the host.

Antibacterial effect of probiotics

Normal microbial population on host surface or the gut will be performing the function of restricting the growth of opportunistic pathogens through production of antimicrobial compounds and competing for nutrients and energy. Probiotic bacteria demonstrate the antibacterial activity by secretion of antibiotics, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, organic acids etc. Antibiotics and bacteriocines inhibit opportunistic microbes while siderophores compete for free iron and enzymes induce bacteriolytic activity.

Competition for Adhesion Sites

Adhesion of microbes to mucosal surface of the host is the primary requirement for the establishment of pathogenic microbe. Possible competition for such attachment sites would reduce the virulence of the pathogens. Though this mechanism has been proved in the laboratory experimental animals and poultry, similar studies in aquatic species are rare. However, the necessity of pathogenic vibrios to adhere to fish mucosa for entry and the ability of probiotic bacteria to adhere to similar sites in fish gut suggest that competition for adhesion sites could be one of the possible mechanisms of probiotic action in aquatic animals also.

Antiviral effect of probiotics

Several studies have suggested the antiviral activity of probiotic bacteria in medical and veterinary microbiology research. Some of the prominent probiotic bacteria to demonstrate antiviral activities are, *Lactobacillus ruminis, L. reuteri, L. rhamnosus, L. casei, Bifidobacterium longum, B. adolescentis, B. adolescentis, and Leuconostoc mesenteroides*. Important human and animal viruses shown to be inactivated by probiotic bacteria are rotavirus, avian influenza (H9N2), transmissible gastroenteritis virus (TGEV), New Castle disease virus, coxsackie virus B3, enterovirus 71, herpes simplex virus type 1 and human papilloma virus type 16, HIV-1. Several mechanisms have been suggested to explain the anti-viral activity of the probiotic bacteria like, induction of cytokine production and bacteriocins, enhancing the antiviral macrophage viability, down regulation of viral virulent gene, inhibiting the adsorption of viral particles or blocking

their entry into cells, modulation of signal transduction pathways involved in any step of the viral cycle and alteration in the host-virus interaction sites [14].

Growth promoting effect of probiotics

Improvement in growth and survival is the function of all the beneficial properties of probiotic bacteria. Antibacterial activity reduces the pathogenic microbial loads while an improved immune system protects against low levels of infections. Further, enzyme secretion in the gut improves digestion while improvement in water quality reduces the stress. Some studies have also suggested that the beneficial effect of probiotic application, at least in larval rearing systems, could also be attributed to microbial cultures acting as source of micronutrients and proteins. Some of the photosynthetic bacteria are rich in proteins, carotenoids, biological cofactors, and vitamins and are known to stimulate growth, improve survival and production in several species of live feed, fish and shrimp [15].

Environmental impact of probiotics

Deterioration of fish pond environment is a normal consequence of culture intensification. Several mitigating approaches, including bioremediation, are being practised successfully. Accumulation of nitrogenous wastes like ammonia, nitrite and nitrate in water column and sulphur compounds in pond bottom are toxic to cultured animals, leading to stress, growth retardation and mortality. Nitrogenous species are mitigated by the application of nitrogen recycling bacteria like ammonia oxidizing bacteria, nitrite oxidizing bacteria and denitrifying bacteria, while the accumulated sulphur compounds in the pond bottom are mitigated by the application of sulphur recycling bacteria like consortia of sulphur oxidizing bacteria. Generally, products containing gram positive *Bacillus* spp. or photosynthetic bacteria transform the organic matter into simple compounds which can be easily utilized by the planktons in the system. Regular application of water and soil probiotics during the culture period prevents the build-up of these toxic substances in the system.

Selection of potential probiotic microbe

The search for probiotic is dynamic and a continuous process, and the list of potential beneficial bacteria keep growing and researchers continue to report renewed understanding of the mechanism of their action in different species of hosts. The principles behind the probiotic product development are:

a. Selection of potential microbe

b. Evaluation of microbe *in vivo* and *in vitro* systems

c. Standardization of dose, schedule and route of application

d. Optimizing the production in bulk industrial scale

e. Development of effective formulations and shelf life

f. Performance of the final product under different culture systems.

In general, the chances of isolating the beneficial bacteria are high if the source is near the site of action and the species of target animal. However, there are several reports suggesting the beneficial effects of bacteria isolated from completely unrelated target system. For example, the *Lactobacillus* spp isolated from bean sprouts reportedly induced immune stimulation, disease protection and higher weight gain ability and survival in olive flounder [17] while host gut environment was also suggested to be a useful source for the development of probiotics [3].

The development of effective probiotic products for application in a complex system like aquaculture needs concerted multi disciplinary approach. Since aquaculture involves rearing of different varieties of fish, shrimp, crabs, mussels, etc, under wide environments like freshwater, brackishwater and marine waters, the probiotic products for each species cultured under different environments need to be studied separately. Though reports say that isolates from completely different environment from the system where the probiotic is supposed to exert its effect also make a good probiotic, it is prudent to isolate probiotic from the host environment. *In vitro* antagonistic test could be a better option to start the screening followed by effect on live food organisms like algae, rotifers and artemia, larval stages of fish, shrimp and molluscs under monoxenic conditions. Initial screening for isolation of potential probiotic bacteria using in vitro antagonistic activity has two major problems. Isolates which show efficient inhibition during *in vitro* studies need not necessarily produce similar effect in actual *in vivo* conditions due to several environmental factors. Further, some of the potential isolates which might have the ability to induce immune stimulation, digestive enzymes and compete for attachment sites and nutrients with pathogen are eliminated for not showing *in vitro* antagonistic activity. Suggested inclusion of experiments using test animals needs higher facility and number of animals [18]. Starting the research in isolates with proven effectiveness in terrestrial animals and human medicine was also suggested to be one of the options though this method does not allow the identification of novel isolates. Finally, the safety of the isolates needs to be confirmed by challenging under normal and stressed conditions through injection, immersion or feed. Ultimately the test for suitability of the isolate will be monitoring growth, survival, immune response and resistance to pathogen challenge.

Basic characters of the microbe to qualify as probiotic bacteria include:

1. Safe to target and non-target hosts at the site of application

2. Stable and active at the site of action

3. Capable of colonizing and proliferating at host site

4. Able to impart the beneficial effect(s) to host or culture environment

5. Absence of virulence or antibiotic resistant genes.

Limitation of probiotic effectiveness

1. Microbes are very sensitive to environmental parameters. Hence observed beneficial effect of the products may vary with culture systems

2. Host and probiotic bacterial interactions are species and strain specific hence the observed beneficial effects of the product may vary with cultured aquatic animal

3. Probiotic bacteria needs to be built up in the system for effective function. Hence regular application of the probiotic products ensures the beneficial effect

4. Gut, water and soil probiotics work at their respective sites in the system, hence they need to be applied through appropriate route

5. Since there will be a competition between probiotic bacteria and opportunistic pathogen, it is necessary that probiotic product needs to be applied in sufficient quantity

Quality standards

Though the beneficial effects of probiotic application are proven, maintaining the quality of the product from the production point to the end use is the real challenge. The economics of probiotic production depends on the yield and viability of the microbes which is dependent on factors like species/strain of microbe, growth conditions like media composition, temperature, pH, oxygen, etc. Further, selection of suitable preservatives to maintain the viability of the microbes in the final formulation is vital. Microbes of genus *Bacillus* are more successful as probiotic product due to their gram-positive and spore-forming nature, making them more stable in powder or crystal formulations.

Most commonly used microbes   as probiotics for aquaculture

Genus	Species
Bacillus	*B. subtilis, B.cereus, B. pumilus, B. licheniformis, B. megaterium, B. polymyxa, B. coagulans, B.amyloliqifaciens*
Pseudomonas	*P. aeruginosa, P.* synxantha
Thiobacillus	*T. thiooxidans, T. ferroxidans, T. denitrificans*
Lactobacillus	*L.plantarum, L.lactis*, *L. acidophilus, L.casai, L.sporogenes*
Saccharomyces	*S. cerevisiae* Sacchromyces boulardi, Sacchromyce cerisia,
Nitrosomonas	*N. europaea*
Nitrobacter	*N. winogradskyi*
Aspergillus	*A.oryzae, A. niger*

Conclusions

Ever increasing demand for probiotic products in aquaculture has put emphasis on research and development in this field of study. The number of probiotic products is extensively used in fish or shrimp farming with mixed results. However, products with valid scientific data need to be developed for effective utilization of these products in aquaculture. Application of probiotics in aquaculture is like an insurance; its effectiveness could be better appreciated in case of stress or disease conditions. It is generally believed that instead of a single strain, mixed culture products would give better result as each isolate might have difference in their ability to establish in host environment and act through different mechanism of action, probably with some synergy. Finally, success depends on economical production on an industrial scale and shelf life of the product under harsh environmental conditions.

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Environmental Probiotics

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With the introduction of artificial breeding, which assured the supply of fish seed, aquaculture got a fillip and now aquaculture is the fastest growing food producing sector in the world which has grown more than 30% between 2006-2011. Present day aquaculture is an intensive farming with inputs of fish seed, feed, medicines and health supplements, and artificial oxygenation of water.

The intensification of aquaculture has brought several diseases and other health problems for finfish and shellfish. Unlike terrestrial animals that are continually exposed to air having low microbial counts, fish constantly live in water, breathe and drink water, take food from water and breed in water where oxygen level varies significantly and the microbial load is very high. In different estimates, aerobic plate count of bacteria of freshwater may range from a few thousands to lakhs, and the total cultivable and non-cultivable fungus, bacteria and virus load may be several orders higher. In the aquatic ecosystem, the host, the pathogen and the environment continually interact closely determining the health of the host and disease outbreaks. Fish and shellfish are poikilothermic animals having primitive and under-developed immune system and are greatly influenced by environmental qualities. In other words, the host-pathogen-environment interaction triad is very large in the aquatic system. Besides, microbial elemental cycling and aquatic production are intricately related and dependent on phytoplankton at the primary producer level and bacteria and fungus at the secondary level. Thus, the role of microbes in the aquatic food chain and fish/shellfish health and diseases are of paramount importance.

For decades, antibiotics were being effectively used in aquaculture for disease management. But unscientific use of antibiotics in human practice and the use of antibiotic as growth promoters in intensive livestock farming have reduced the effectiveness of these drugs. Issues like the emergence of antibiotic-resistant strains and their transmission to other pathogens, reduction in the efficacy of antibiotics are causes of concern in the last decade. This development has reduced the options of antibiotic usage in aquaculture and imposed the necessity of alternative therapies. As benign and healthy alternative, probiotics are now increasingly used for fish health promotion and disease prevention. Initially, the scope of probiotics was limited only to "beneficially affect the host by improving its intestinal microbial balance" [1]. However in subsequent decades researchers have identified several other benefits of probiotics and now-a-days live microbes and microbial

products are used in fish and shellfish farming for various purposes, such as enhancing host immunity, pathogen inhibition, stress tolerance, growth promotion, enhanced feed digestibility and nutrient availability and maintaining water quality. As such, the scope of probiotics have been broadened to preparations that impart "beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of feed or enhancing its nutritive value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" [2]. A wide range of microbes have been used as probiotics, viz., *Lactobacillus* spp., *Lactococcus* spp., *Saccharomyces* spp., *Bacillus* spp., *Carnobacterium* spp., *Streptococcus* spp., *Micrococcus* spp., *Leuconostoc* spp., Roseobacter, *Nitrosomonas* spp., *Nitrobacter* spp., *Pseudomonas* sp., *Bifidobacterium* spp., *Streptomyces* spp., *Pediococcus* spp., *Leuconostoc* spp. etc. The use of classical probiotics acting on the host for growth promotion, digestion and disease resistance have been extensively reviewed and we limit the scope of this chapter on the use and prospective use of microbes for water productivity enhancement and improvement of water quality. These water probiotics, when applied in water, multiply and interact with culture environment and host, and modify bacterial composition of water and sediments towards reduced organic matter and nutrient load, better water quality, improved algal growth, suppressed cyanobacterial bloom, less pathogen load, greater survival and improved production.

Probiotics in nitrogen cycling

Nitrogen is one of the macro-elements that critically determine aquatic health and productivity. Like in land-based agriculture, nitrogen is one of the deficient nutrients limiting primary productivity in aquatic environments. However, unlike in agriculture, the use of urea/ chemical nitrogen fertilizer is limited and also not advised in aquaculture. Rather, the use of nitrogen-fixing microbes and ferns has been suggested to enhance nitrogen balance in aquatic systems. Examples include azolla, blue green algae, aerobic *Azotobacter* sp. and anaerobic *Clostridium pasteurianum*. Nitrogenase is the key enzyme in nitrogen fixation. Nitrogen fixation in aquatic environments has traditionally been considered a minor source of new nitrogen, but the recently discovered abundance of diazotrophic (nitrogen fixing) organisms in various aquatic habitats suggests greater role of biological nitrogen fixation in total nitrogen budget in aquaculture.

The present day aquaculture is highly intensive and least dependent on primary productivity or carrying capacity of the ecosystem and the use of feed is the mainstay. Regular use of feed has tremendously increased the nitrogenous wastes in the aquaculture system. For example, in raceway trout farm the total Kjeldahl nitrogen increases up to 6.4 mg/l in 7 months period [3]. This nutrient increase is even higher in shrimp farms. The organic nitrogen compounds in unused feed and animal excreta are microbially

converted to highly toxic ammonia and nitrite. In one estimate [4] in a recirculating tilapia production system, the total ammonia increased from 4.73 to 14.8 mg/l and the nitrite level increased from 3.75 to 9.77 mg/l in 3 weeks period. It is estimated that about 50.4g of ammonia nitrogen is generated for every kilogram of feed, containing 35 percent protein, used in aquaculture.

Of the total ammonia, un-ionized ammonia is most toxic to aquatic organisms as it can readily diffuse through cell membranes and is highly lipid-soluble. Nitrite is also one of the toxic forms of nitrogen. Although the susceptibility of different cultured species vary to high concentrations of ammonia, nitrite and nitrate, high concentrations of these nitrogenous compounds affect animal health and likely cause high mortality. The use of probiotics, especially nitrifying bacteria, has been effective in reducing the ammonia and nitrite levels and thus improves water quality. As such, nitrifying bacteria are the most widely used probiotics to remove toxic ammonia and nitrite and improve water quality in aquaria and aquaculture systems.

Since the classical work of Winogradsky [5] there have been enormous works on nitrifying bacteria in the nitrogen cycle. The aerobic autotrophic *Nitrosomonas* obligately oxidizes ammonia to nitrite by ammonia monooxygenase enzyme and *Nitrobacter* further mineralizes nitrite to significantly less toxic nitrate by nitritase enzyme. These bacteria form biofilms by virtue of their polymer excretion and are thus effectively used in biofilters. Most of the commercial probiotic preparations for water quality enhancement incorporate these nitrifying bacteria. Denitrifying bacteria like *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Cellulosimicrobium*, *Halomonas*, *Microbacterium*, *Paracoccus*, *Pseudomonas*, *Sphingobacterium* and *Stenotrophomas* reduce nitrate or nitrite to N_2 and have been identified for use as probiotic in aquaculture systems to ameliorate nitrite and nitrate accumulation [6]. Besides microbial products, substances derived from plant sources, including yucca extract, ricinoleate, tannic acid and citrus seed extract have also been used in culture systems with considerable improvement in water quality [2].

Probiotics in organic matter decomposition

In intensive and semi-intensive systems, high stocking densities of fish along with excessive feeding often leads to deterioration of water quality and proliferation of pathogens. In some cases, entry/use of sewage water cause heavy organic matter loading. In aquaculture systems there is huge accumulation of organic matter, in both water column and sediment. Build up of dissolved, as well as particulate organic matter may promote bloom formation and other water quality aberrations, including low dissolved oxygen, toxic ammonia, H_2S and methane gas accumulation. A number of studies have observed that use of probiotics, especially *Bacillus licheniformis* and *B. subtilis*, were effective in reducing the organic matter load and maintaining water dissolved oxygen, total ammonia,

nitrite, phosphate and pH in acceptable ranges [7-9] both in finfish and shrimp cultures, besides increase in beneficial microbiota, reduced pathogen load/pathogenicity and higher fish/shellfish survival. Similar reduction in inorganic nitrogen and phosphate levels and increase in beneficial microbiota have been observed in *Penaeus vannamei* shrimp farms from use of commercial probiotic preparation containing *Bacillus* sp., *Saccharomyces cerevisiae*, *Nitrosomas* sp. and *Nitrobacter* sp. [10]. *Bacillus* spp. has been shown to reduce chemical oxygen demand [11].

In *Penaeus monodon* culture the use of bacterial products showed reduction in organic matter and total sulphur contents in pond sediments suggesting enhanced mineralization and sulphur cycling activities by heterotrophic bacteria [12]; the water quality was within limited range suitable for shrimp growth. It has been suggested that members of the genus *Bacillus* are more efficient in complete mineralization of dissolved and particulate organic matter to CO_2, thus promoting more stable phytoplankton growth than gram negative bacteria that converts organic matter preferentially to bacterial biomass and slime [2]. Whole genome sequence studies have identified a suite of simple to complex organic matter degrading enzymes in *Bacillus licheniformis* and *B. subtilis* that make them effective organic matter degraders [13]. Further, *B. licheniformis* has polyphosphate accumulating system that makes it an effective phosphate quencher in oxic environments [13] and thus might be instrumental in regulating phosphorus availability. Other than *Bacillus*, members of the genera *Pseudomonas, Acinetobacter, Cellulomonas* etc. efficiently degrade organic matter, including toxic pollutants and thus regulate microbiota composition, including pathogen load in the aquatic system [14]. It has been observed that commercial probiotics caused marked changes in heterotrophic bacteria and phytoplankton, improving the environmental quality of water and sediment [15]. However, there are contradictory studies that failed to record significant water quality improvements, other than a reduction in ammonia and nitrite levels, from use of probiotics [2, 16].

Besides water quality, probiotics have been found to enhance FCR and act as growth promoter in fish, possibly through multiple actions like providing nutrients like vitamins, detoxifying feed ingredients, enhancing nutrient digestibility as well as improved microbiota and water quality [17].

Probiotics in phosphorus availability

Like nitrogen, phosphorus (P) is another nutrient that critically determines trophic state and productivity. In some freshwater environs like lakes, reservoirs, ponds/tanks, and canals, the levels of available form of P may be low limiting the phytoplankton growth, while in those receiving organic rich sewage, feed, etc. the level may be very high, triggering eutrophication.

Microbes are intricately involved in P recycling and enhance P availability in an eco-friendly manner. In aquatic environment P remains in various forms like dissolved inorganic P, dissolved organic matter, particulate organic matter, and forms sorbed to the soil. In sediment P remains mostly sorbed with bivalent cations viz. Ca, Mn, Al and Fe or complexed with organic matter. Phosphorus solubilizing microbes release a substantial part of Ca-bound P from sediment and have been detected both in freshwater and marine environments [18]. Besides inorganic P pool, substantial part of organic bound sediment P is also released through microbial mineralization and is responsible for summer eutrophication. Microorganisms, often in consortium, degrade the sediment organic matter and releases P through a cascade of enzymes like cellulases, phytases, phosphatases, nucleotidase, etc. Several of these microbes are efficient phytase enzyme producers and their use as feed probiotic has been tested. Among several genera, members of the genus *Bacillus* are the dominant microbes in the sediment P release process [18] and might play critical roles in nutrient management and the carrying capacity of the ecosystem. There is significant scope of use of these microbes as biofertilizer in aquaculture systems, other than as feed probiotics.

Probiotics in sulfur cycling

Sulfur is part of organic matter and the regular use of feed enhances the sulfur load in aquaculture systems. Microbial mineralization of organic sulfur or reduction of sulfate leads to production of toxic hydrogen sulfide, which may again be microbially oxidized to sulfate. Oxygen availability plays a great role in S oxidation and reduction. In aquatic environments sulfate and hydrogen sulfide are constantly recycled between oxidation and reduction steps; sulfate reduction is more prominent in pond bottoms, characterized by depleted nitrate and sharp redox decline. Oxidation of toxic hydrogen sulfide to less toxic sulfate is carried out by sulphur oxidizing bacteria like *Thiobacillus*, *Thiomicrospira*, *Rhodovulum imhoffii*, *Pseudoxanthomonas* sp., *Paracoccus denitrificans*, *Roseobacter* sp., *Rhodobacter* sp. etc. [19], and are important candidates for maintaining water quality in aquaculture, especially in shrimp culture systems.

Future perspectives

In present day aquaculture, probiotics are becoming popular for better survival, growth, feed efficiency and higher production. However, the mechanism of action of probiotics are little known and several of the microbes used in commercial probitotic preparations have been derived from terrestrial and other environments without much study on their survival, growth, efficacy as well as conversion to virulent form in aquatic environments. Although the use of probiotics may limit the use of antibiotics in aquaculture, long term effects of regular use of extraneous bacteria, in form of probiotics, needs evaluation from ecological, environmental and human and animal safety perspectives.

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11. Biological Control

Phage therapy in aquaculture

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Aquaculture is the fastest-growing food production sector with an average annual growth rate of 7.5% in the last two decades. Along with capture fisheries, it supplies 17% of animal protein and support the livelihood of 12 % of the world's population. The global aquaculture production in 2014 recorded 74 million tonnes with an estimated value of US$160.2 billion [1]. India stands taller in the sector with 2nd position and contributes 7.3% of the global aquaculture production. The sector plays crucial role in generating foreign exchange through export and provides a employment and livelihood to rural population. India has diverse water resources from a large riverine pool to a vast coastal belt. Among all the aquaculture produce, shrimp is the most valued commodity and accounts for 66.1% of the total earnings from seafood export.

The greatest threats to sustainable aquaculture are biological (pathogens and parasites) and chemical agents (antibiotics, agro-chemicals and organic pollutants). Aquaculture frequently suffers heavy losses due to microbial diseases of viral, bacterial and parasitic origin that threaten the growth and sustainability of this sector. Since its emergence, white spot disease (WSD) posed a serious challenge to the global shrimp aquaculture, with mortality often reaching 100% within 3-5 days of infection. The WSD and other diseases such as monodon slow growth syndrome and loose shell syndrome of farmed black tiger shrimp significantly slowed down aquaculture development in India during the last decade leading to the introduction of exotic specific pathogen free (SPF) Pacific white shrimp, *Penaeus vannamei* in the year 2009. However, recent years witnessed the widespread emergence of microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) which poses serious challenge to *P. vannamei* culture due to its growth retardation effect. Many Asian countries, except India, witnessed the devastation of their flourishing shrimp industry due to early mortality syndrome (EMS) / acute hepatopancreatic necrosis disease (AHPND) caused by a new strain of *Vibrio parahemolyticus*. These diseases pose serious threat to aquaculture industry and demands new line of remedial measures for prevention and cure.

Bacterial diseases in aquaculture

Despite advancements in better management practices, bacterial infections still pose a major problem in both hatcheries and grow-out culture, often causing significant mortalities. Bacterial infection in aquaculture system is typically associated with pathogenic

Vibrio spp., Aeromonas spp., Pseudomonas spp., Edwardsiella spp. and *Streptococcus spp*. Vibriosis is considered as the significant bacterial infection in the brackishwater sector, especially in shrimp hatchery, causing up to 100% mortality during mysis and early post-larval stages [2]. Luminescent vibrio such as *V. harveyi* has been mostly reported to be responsible for vibriosis in larval shrimp. In recent years, *V. campbellii* has also been found to be frequently associated with vibriosis infection in larval shrimp and now being considered as an emerging pathogen. Recently, in grow out shrimp farms, *V. parahemolyticus* has become a dreadful pathogen, causing AHPND, popularly known as EMS. It was first reported from grow out *P. vannamei* farms in China in the year 2009. The impact of EMS was such that China lost almost 80% of its shrimp production [3]. Very high economic loss was also reported in Vietnam and Thailand with severe mortality till 2014. The disease is caused by bacterial pathogen *V. parahemolyticus* which carry toxin producing lysogenic phage. Many other *Vibrio* species such as *V. campbellii*, *V. mimicus*, *V. anguillarum*, *V. vulnificus* and *V. alginolyticus* have been widely reported from marine shrimp and fishes, and considered as serious pathogens of aquaculture importance.

In freshwater system also bacterial diseases are a major concern. *Aeromonas hydrophila, Edwardsiella tarda, Streptococcus iniae* are some of the major pathogens in freshwater system. *A. hydrophila* is pathogenic for fish and various reports suggest that it has zoonotic importance as well. The affected fish develop ulcer, tail and fin rot, and hemorrhagic septicaemia with haemorrhages in the gills and anal area. *Aeromonas* spp cause gastroenteritits with symptoms similar to cholera in children and immunocompromised individuals. Another important pathogen is *E. tarda,* a Gram-negative bacterium. It affects a wide variety of fishes in freshwater, brackishwater and marine system and is considered a pathogen of serious zoonotic importance. In eel and catfish it causes edwardsiellosis which is also known as emphysematous putrefactive disease of catfish or fish gangrene. The other major bacterial pathogens are *Streptococcus iniae* and *Pseudomonas aeruginosa,* which apart from causing diseases in aquaculture are also serious human pathogens.

Preventive and therapeutic measures against bacterial pathogens in aquaculture

A variety of antimicrobials, vaccines, probiotics and immunostimulants are being used as therapeutics and preventive measures to control and treat bacterial diseases in aquaculture. Historically, antibiotics have been widely used for treating bacterial pathogens. In aquaculture system also, antibiotic such as tetracycline has been widely applied in the past to treat bacterial infection. However, due to indiscriminate and widespread use of antibiotics, most of the bacterial pathogens are now resistant to one or more number

antibiotics. The condition further deteriorated by the evolution of superbugs, which are multiple drug resistant (MDR) such as *Staphylococcus aureus* or extremely drug resistant (XDR) and total drug resistant (TDR) strains of deadly pathogen *Mycobacterium tuberculosis* [4]. As discovery of new line of antibiotics is a very slow process, extreme pressure is on scientific community, policy makers and public advocates to reduce and ban the use of antibiotics other than for therapeutic purpose of human and animals. Due to strict guidelines adopted by USA and other western countries, even the trace of antibiotics in nano to pico gram level in farm produce lead to rejection and destruction of whole lot of exported product. Use of antibiotics in aquaculture is hazardous from ecological and health perspective, as seeing its vastness the antibiotics dose will always be at sub-therapeutic level, an ideal condition for development of antibiotic resistance. This could be substantiated from the fact that multi-resistant strains are more common in fish farms compared to that in the nearby coastal areas [5]. In another study it was reported that in the marine environment, most (90%) bacterial strains are resistant to more than one antibiotic and 20% are resistant to at least five antibiotics [6]. Moreover, antibiotics not only destroy the targeted pathogenic bacterium but also destroy the beneficial microflora in intestine of fish. This disturbs the ecological balance and microbial biodiversity. Due to concern over emerging antibiotic resistance and restrictions imposed on the use of antibiotics by several countries, there is an urgent need for the development of therapeutic technologies alternative to antibiotics in aquaculture. The therapeutic and prophylactic approaches such as vaccines, probiotics, immunostimuanlts and phage therapy are considered most promising and need of the hour.

Bacteriophages

Bacteriophages are ubiquitous in our world—in the soil, rivers and oceans including deep sea vents. Bacteriophages are viruses that specifically infect and replicate within a bacterium. They are made up of protein capsid which encapsulates either DNA or RNA as genetic material. In short, they are referred to as 'phage'. It was first reported by Fredrick Twort (1915) and Felix D' Herelle (1917). D'Herrele (1919) later reported that phages always appeared in patient recovering from *Shigella dysentery*, and successfully employed phage therapy for the treatment of severe dysentery [7].

i. Structure of Phages

Bacteriophages exist in three basic structural forms, namely icosahedral (20 sided) head with a tail; icosahedral head without a tail, and a filamentous form. A typical myovirus has a head encircling genetic material DNA, neck, tail and tail fibers (Fig 1).

Bacteriophage Structure

Figure 1
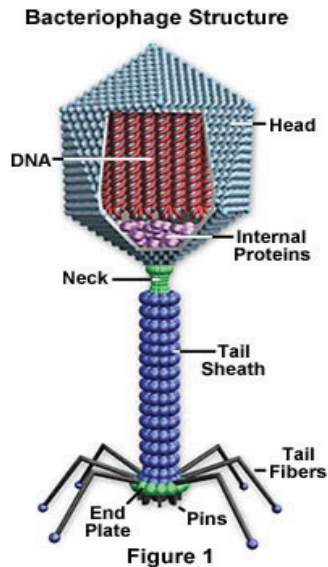
ii. Classification of Phages

Phages have been classified into 13 families based on the morphology, the type of nucleic acid, and the presence or absence of envelope. About 96% reported phages are tailed phages which have icosahedral head, tail and DS DNA as genetic material. These tailed phages have been classified under order Caudovirales and have three families: Myoviridae (contractile tail with neck; eg KVP20), Siphoviridae (long non-contractile tail; eg λ phage) and Podoviridae (extremely short non-contractile tail; eg T7). The other phages, which are classified into ten families, constitute only 4% of the total. These are cubic, filamentous, or pleomorphic. Most of the therapeutic phages are tailed phages.

iii Bacteriophage life cycle

Based on the life cycle, phages are of two types, lytic and lysogenic. The life cycle of lytic phages involves:

a. Adsorption: The virus attaches to the cell wall surface of bacterium.

b. Penetration: Entry of phage DNA into the host cell.

c. Host-mediated replication of phage components including capsid proteins and nucleic acids.

d. Assembly of new phage particles.

e. Lysis of bacterial host.

f. Release of progeny phages.
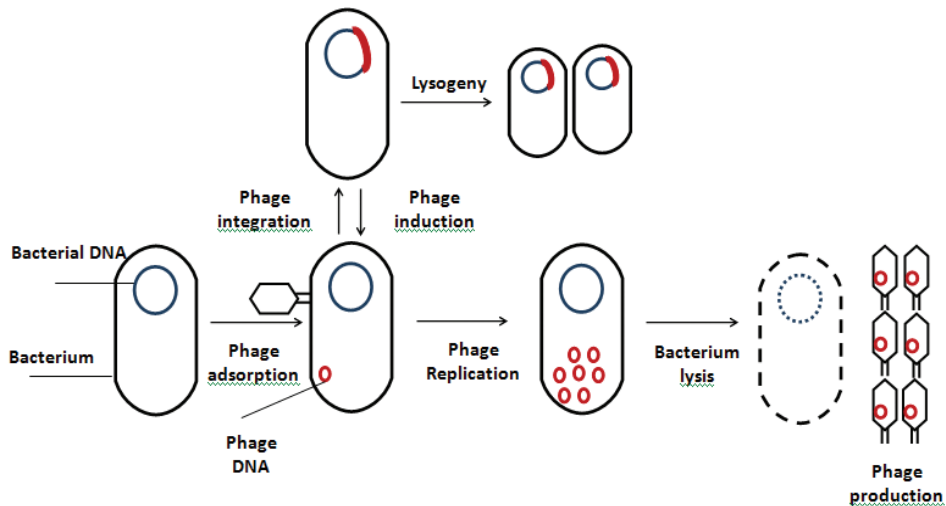
Fig. 2. Lytic and lysogenic life cycle of bacteriophage

Lysogenic phages have the potential to integrate their DNA into the host's genome. Phage lysogenized host cells replicate normally for generations without causing any harm to the host cells. However, sometimes they excise the phage DNA, either spontaneously or through induction by chemicals, radiation, carcinogens, etc. and synthesize new phage particles, which in turn lyse the host, releasing more lysogenic viruses into the surrounding medium.

Table 1. Difference between lytic and lysogenic phages

Character	Lytic phage	Lysogenic phage
Integration of phage genome with bacterial host	No	Yes
Role in bacterial virulence	No	Yes
Transfer of bacterial virulence from one bacteria to another	No	Yes
Role in antibiotics resistance gene transfer	No	Yes

Character	Lytic phage	Lysogenic phage
Multiplication	Always result in lysis	Generally don't lyse bacterial cell
Application in phage therapy	Yes	Never

Phage therapy

Ernst Hankin in 1896 for the first time reported the presence of marked antibacterial activity (against *Vibrio cholerae*) in the waters of Ganga and Yamuna Rivers in India. However, the bacteriophages for the first time were discovered by Frederick Twort (1915) and Felix d'Hérelle (1917) [7]. Phages are the natural killer of bacteria. Phage therapy represents the application of phages for the treatment of bacterial infections. It was introduced by Felix d' Herelle [7] in 1920, 20 years before introduction of the first antibiotics, penicillin. At the time of discovery, phage therapy was considered as a possible treatment method against bacterial diseases. However, its therapeutic use remained limited in the former Soviet Union and Eastern Europe and was abandoned by the rest of the world with the arrival of the antibiotic. However, the emergence of bacterial multidrug resistance has motivated the scientific community to re-evaluate phage therapy for bacterial infections.

i. History of phage therapy

Most of the experiments on phage therapy were done in Eastern Europe and the Soviet Union to treat human cases. During World War II, the Soviet Union used bacteriophages to treat many soldiers infected with various bacterial diseases like dysentery and gangrene [8]. In Eastern Europe phage therapy continued without interruption in centres such as the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi, Georgia, and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland. For 80 years Georgian doctors have been successfully treating local people, including babies and new-borns, with phages [8]. Another successful example of phage therapy was Staphylococcal phage lysate (SPL), licensed for use in humans until the 1990s, and was administered intranasal, topical, oral, subcutaneous and intravenous with only minor side effects.

ii. Desirable quality of phage for therapy

a. Must be a lytic phage: Phages used for therapeutic applications must be carefully scrutinized to ensure that they are lytic phages. Lysogenic phages have been shown to enhance the virulence of pathogens, as in the case of *V. harveyi,*

V. parahemolyticus and *V. cholerae*. Further, they also have the potential to transfer antibiotic resistance gene through horizontal gene transfer. Therefore, lysogenic phages should never be used for therapeutic purpose.

b. Broad spectrum phage: Phages capable of infecting multiple strains within a species is the most desirable quality required for selecting a phage for therapeutic purpose.

c. Phage cocktails: Mixtures or cocktails of phages with different host specificities may be useful to prevent the development of phage resistant pathogens. It has been suggested that to reduce the likelihood of the development of phage resistance, different combinations of phages should be used each year on farms. Cocktails of phages are widely viewed as a practical approach to combating phage resistance while providing an effective treatment against a range of pathogens or strains.

d. Phages with larger burst size and shorter latent period should be selected. Burst size denotes the number of phage particles released from a single infected bacterial cell. Phages with larger burst size and shorter latent period multiply very fast and control bacterial pathogens faster at lower doses.

e. Phages should have longer survival in aquaculture condition. This will critically decide the dose, frequency of dose and the final outcome. Therefore, before field application phages should be tested for survival against a range of environmental parameters such as salinity, temperature, pH, dissolved oxygen, organic matter, nitrogenous compounds (ammonia, nitrite, nitrate) etc.

iii. Advantage of phage therapy

a. Alternative to antibiotics therapy: Historically, antibiotics have been widely used for treating bacterial pathogens. But due to emergence of antibiotics resistance and strict guidelines for their application in aquaculture, search for alternative therapeutics for bacterial pathogens is the necessity of time. Phages are natural killer of bacteria, so serves as strong alternative for antibiotics.

b. High level of safety for host as well as beneficial bacteria: Phages are more specific than antibiotics as they kill only specific strains or a particular species of bacteria. Antibiotics are broad spectrum, and kill pathogenic as well as beneficial bacteria. Phages do not act against gut microbial flora which secrete digestive enzymes and enhances digestibility in shrimp and fishes. Further, phages have no harmful effects on eukaryotic organism.

c. Low effective dose required: Phages are capable to replicate in vivo. Therefore a smaller effective dose is required for treatment.

d. Phages are self-dosing: Phages multiply only in the presence of the target bacteria and cannot multiply in its absence. Therefore, they get eliminated from the environment/shrimp/fish once the bacterial host is eliminated.

e. Biofilm therapy: Most of the antimicrobials are ineffective against biofilm due to thick extracellular matrix and dormancy of bacterial cells within biofilm. Phages have the ability to lyse bacteria present in a biofilm and are far more potent than antibiotics. Further, T7 phages have been engineered to overexpress Dispersin B and such phages are 100 times more efficient than normal phages in treating biofilm infection.

f. Combination therapy: A synergistic relationship has been demonstrated between phages and antibiotics. Dual phage-antibiotic therapies could lead to a reduction in the emergence of antibiotic resistant strains.

iv. Challenges in phage therapy

a. Need of cocktail or mix of phages: Bacteriophages have very high bacterial strain specificity. Thus it becomes necessary to make different cocktails of phages for treatment of the same infection because pathogenic bacterial strains differ from region to region.

b. Maintenance of phage bank: Though the chances of resistance in phages are much lower compared to antibiotics, the emergence of resistance has been reported. Therefore, a phage bank needs to be maintained.

c. Treatment against multiple pathogens: Aquaculture is a dynamic system and often a disease involves multiple pathogens. In such cases broad spectrum phages, phage cocktail and start of therapy in the early stage of infection will help.

d. Phage resistance: Bacteria can evolve different receptors either before or during treatment; this can prevent phages from completely eradicating bacteria. However, this can be overcome with cocktail of phages.

e. Lysogenic phage: Many of the phages have genetic element which help them to integrate with host genome. Such phages are called lysogenic phages and play crucial role in the virulence of pathogenic bacteria such as *Vibrio harveyi, V. parahemolyticus,* etc. These phages are also capable to transfer of antibiotic resistant genes among bacteria. Therefore, in no circumstances can a lysogenic phage be employed for phage therapy. Using molecular tools, all analysis should be done that a phage is purely a lytic phage and has no genetic elements required for the recombination process.

f. Bacteriophage production: For human use phage needs to be free from endotoxins and pyrogens which are generated as parts of phage production cycle. However such issues are seldom encountered in the case of aquaculture pathogens.

v. Phage therapy on commercial scale

Eli Lilly is the first company in the field of phage therapy which brought seven phage products in 1930 for human use, to treat a wide range of conditions including abscesses, suppurating wounds and respiratory tract infections. At present, several commercial phage products are available in the market with the approval of EPA, USDA, and FDA. Products targeted against *Listeria monocytogenes* (ListShield™ and LISTEX™ P100) are used as sterilizing agents for processed foods [9]. These are phage cocktails which has acquired Generally Recognized as Safe (GRAS) status from the FDA. Another approved product is AgriPhage by Omnilytics which treats crop pathogens such as *Xanthomonas campestris pv. vesicatoria* and *Pseudomonas syringae* in tomato. The companies involved in phage therapy are listed in Table 2.

Table 2. Commercialisation of phage therapy

Company	Product	Stage of development
JSC Biochimpharm, Georgia	Phage lysates for intestinal problems, for example, dysentery, salmonellosis, dyspepsia, colitis, enterocolitis and for bacterial infections.	Phage tablet
Biopharm L Limited, Georgia	Pyobacteriophage and Intestinal bacteriophage that are mixtures of phage lysates for bacterial intestinal and infection control.	Liquid and tablet phage are sold in the market as over the counter drugs (drugs can be purchased without prescription).
EBI Food Safety Netherlands	LISTEX P100™	Cocktail of phage against *Listeria* in food safety
Intralytix USA	ListShield™	Product contains 6 lytic phages which kill *Listeria monocytogenes* in food.

Company	Product	Stage of development
Intralytix	Ecoshield™	Product available for control of *E. coli* O157:H7
Omnilytics	Agriphage™	Product available for control of *Xanthomonas campestris*
AmpliPhi biosciences Corp UK	BioPhage-PA	Treatment against *Pseudomonas aeruginosa* in chronic ear disease
CheilJedang Corporation	BioTector	Control of *Salmonella* in poultry
Micreos Food Safety	SALMONELEX™	Salmonella infection in food
Omnilytics	BacWash™	Target *Salmonella* and *E. coli* O157:H7.
Viridax, USA	Viridax ™	Treat *S. aureus*
BioControl, UK	Whole Phage	Phase II trial completed for treating *Pseudomonas* infections of ear
Blaze Venture Technologies UK	Phage immobilisation technology.	Licensed for use for methicillin resistant *S. aureus* (MRSA)
Gangagen India	Whole phage	Phage trial under preclinical stage against *S. aureus*

Route, dose and delivery of phages

The route of administration is likely to affect the therapeutic outcomes. Phage therapy seems somewhat easier in aquaculture than for human or terrestrial animals, as live fish can be treated via their feed, by injection, by immersion in water containing the phages or by swabbing of surface wound. Surface swabbing with phages was found effective for treating ulcerative skin lesions caused by *P. aeruginosa* in catfish [10].

However, for deep and systemic infections, phage can be employed through injection as in the case of fish brooder. Multiple phage treatments via feed is another possibility which may enhance therapeutic efficacy over single treatment.

Phage dosage is likely to be a major factor in the effectiveness of treatment and cost of therapy as well. A wide range of doses, mostly ranging between 0.01 to 100 multiplicity of infection (MOI), has been applied in the laboratory and field testing. For an example, an MOI of 100 was used to treat induced vibriosis by *V. alginolyticus* in *Artemia salina* [11] while an MOI of 0.01 totally eliminated the symptoms of columnaris disease in catfish [12].

Phages can usually be freeze dried and turned into pills without impacting its efficacy. In pill form it can be maintained at room temperature for more than an year. Other forms of administration include application in liquid form. Liquid forms are usually best kept refrigerated. In case of human or terrestrial animal, oral administration works better when an antacid is included, as this increases the number of phages surviving the passage through the stomach. But this has to be scientifically validated in fishes.

For better phage delivery and to extend their viability, microencapsulation of phages has been suggested. Microencapsulation could also be designed for timed release of phages at a controlled rate to optimize their persistence and effectiveness. Several reports have suggested that oral microencapsulated forms for bacteriophages remain bioactive in a simulated gastrointestinal tract environment [13, 14]. Chitosan-alginate and poly ethylene glycol (PEG) are being tried for this purpose.

Phage therapy against important aquaculture pathogens

i. *Vibrio harveyi*

Vibriosis is a serious problem in shrimp hatchery where it causes mass mortality in mysis and early postlarval stages, due to luminescent vibrios such as *V. harveyi* and the emerging pathogen, *V. campbellii*. The work conducted at ICAR-CIBA revealed that vibriosis-infected hatcheries often harbour bacteriophages specific for *V. harveyi* which is predominantly associated with maturation tank and spawning tank water [15]. The four isolated lytic bacteriophages (φVh1, φVh2, φVh3, and φVh4) possessed broad spectrum infectivity against luminescent *V. harveyi* isolates. The three phages belonged to siphoviridae and one to podoviridae [16]. ICAR-CIBA is standardising the mass production of these phages to use as therapeutic agents against vibriosis. Some reports suggest that phage therapy could be a potent weapon against virbiosis in shrimp hatchery. Vinod et al. [17] reported that a lytic siphovirus, isolated from shrimp farm, was effective against all the 50 tested *V. harveyi* isolates. At laboratory scale, the phage therapy recorded 3 log

reduction in bacterial count with better postlarval survival (80%) compared to control (25%). A trial was also conducted in hatchery where they observed better postlarval survival against vibriosis by phage therapy (86%) compared to antibiotics (40%) and control (17%). Later on the same group [18] tested 4 lytic phages which were able to lyse 55-70% of total tested 100 isolates. These phages were also able to control *V. harveyi* population in biofilm.

ii. *Vibrio parahemolyticus*

V. parahemolyticus has emerged as a serious pathogen in aquaculture, especially in the shrimp industry after the emergence of AHPND. The disease has devastated the flourishing shrimp industry in many Asian countries such as China, Thailand, Vietnam etc. Recently Martínez-Díaz and Hipólito-Morales [19] evaluated the phage therapy against induced vibriosis in *Artemia franciscana*. A single dose of vmps1 phage was effective to control *V. parahemolyticus* infection in brine shrimp. However, they observed that phage therapy was ineffective when the application of phages was delayed. In another experiment, Lomelí-Ortega and Martínez-Díaz [20] evaluated the effectiveness of phage therapy in controlling *V. parahemolyticus* induced virbiosis infection in *P. vannamei*. The results showed that lytic phages (A3S and Vpms 1) were effective even at low doses (0.1 MOI) in reducing the mortality. The delayed phage applications (6- h post infection) was also capable to reduce the mortality and progress of the infection. Mateus et al. [21] reported that the addition of a bacteriolytic enzyme lysozyme during phage therapy had additive effect in controlling *V. parahemolyticus* infection.

iii. *Vibrio alginolyticus*

Recently two works have been reported on phage therapy against *V. alginolyticus*. Kalatzis et al. [11] reported the application of two phages (φSt2 and φGrn1) against *V. alginolyticus*. The study revealed that *Vibrio* population decreased by 93% in *A. salina* after phage treatment. However, the study used a very high dose of 100 MOI. Sasikala and Srinivasan [22] reported high efficacy of VP01 lytic phage against *V. alginolyticus* and its biofilm. The phage was able to lyse all the tested strain of *V. alginolyticus* and maintained its efficacy at broad range of pH and temperature.

iv. *Vibrio anguillarum*

Vibrio anguillarum is a marine Gram-negative bacterium. It causes fatal hemorrhagic septicemia in more than 50 fresh- and seawater fish species. Recently, Higuera et al. [23] isolated 6 broad host range phages which were able to lyse both *V. anguillarum* and *V. ordalii* but not *V. parahaemolyticus* strains. The treatment using one of the phage strain (COHED) improved the *Salmo salar* survival to 100% against control

(10%). Silva et al. [24] reported that phage application directly in water of the larviculture system improved the survival against vibriosis induced by *V. anguillarum*.

v. *Aeromonas hydrophila*

Aeromonas hydrophila is a Gram-negative bacterium causing tail and fin rot and hemorrhagic septicaemia, a serious bacterial infection in freshwater fishes. In one of the first works in phage therapy in aquaculture, Wu et al. [25] isolated and characterized 8 bacteriophages against *A. hydrophila*. The phage with the strongest lytic activity, AH1, was used to treat *A. hydrophila* infected loaches (*Misgurnus anguillicaudatus*). The co-incubation of phage and pathogen for 3 to 12 h before injection did not cause infections or mortalities at 0.01 MOI against 100% infection and 65% mortality in control. Later Hsu et al. [26] used phage therapy to treat *A. hydrophila* infection in unfiltered fish pond water. The treatment was successful in reducing 99% of the *A. hydrophila* within 8 h with MOI of 0.23. Some phage resistant strains also developed over time. Recently, Jun et al. [27] used 2 myoviruses (pAh1-Cand pAh6-C) to treat *A. hydrophila* infection in loach, *M. anguillicaudatus*. Results indicated that phage therapy provided protection by both intraperitoneal injection or through the food. In a safety trial experiment, healthy loaches were treated with phages at a concentration of 10^{10} PFU/loach fish. Treated fishes did not record mortality or change in physical condition even after one month reflecting that phage therapy is safe even at exceptionally high doses.

vi. *Edwardsiella tarda*

Edwardsiella tarda is the causative agent for edwardsiellosis, also known as enteric septicaemia of catfish (ESC) or emphysematosis putrefactive disease of catfish (EPDC) in a variety of freshwater and marine fish. Wu and Chao [28] identified a phage (ϕET-1) and demonstrated 3 log reduction of *E. tarda* by 0.08 MOI in 8 h. They also evaluated survival of loach (*Misgurnus anguillicaudatus*) with phage therapy. Incubation of *E. tarda* with phage (ϕET-1) at 0.1 MOI for 8 h before injection provided 90% survival in loaches against 5% survival with 2 h incubation. In another study, Hsu et al. [26] evaluated bacteriophages and reported 1.5 log reduction of *E. tarda* with 1 MOI in 8 h. However, the authors did not observe reduction in *E. tarda* titer at 0.1 MOI.

vii. *Lactococcus garvieae*

Lactococcus garvieae formerly known as *Enterococcus seriolicida* and *Streptococcus* sp., causes lactococcosis in fresh water and marine fishes. Park et al. [29] isolated a lytic siphovirus phage (PLgY) from diseased fish. Nakai et al. [30] evaluated the effectiveness of PLgY against *L. garvieae* in yellow tail (*Seriola quinqueradiata*) by i.p injection. At 0.1 MOI, the effectiveness of treatment was better when phages were

administered at the time of bacterial challenge (100% survival) compared to when phages were administered after 1 h (80%) and 24 h (50% survival) after *L. garvieae* injection. They further reported that PLgY phages (PLgY-1, PLgY-16 and PLgY-30) persisted in unsterilized seawater at high levels only for 3 days compared to sterilised autoclaved water (seawater, artificial seawater, and distilled water) where it persisted at high levels for 8 weeks.

viii. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, motile, rod-shaped bacterium that is ubiquitous in the marine environment. It is a common human pathogen and has been reported to infect fish. An Indian study evaluated a lytic phage PT2, isolated from sewage, to cure ulcerative lesions in freshwater catfish (*Clarias gariepinus*) caused by *P. aeruginosa* [10]. Twenty *P. aeruginosa* isolates were obtained from cat fish lesions. These isolates showed multiple drug resistance along with an isolate resistant for carbapenem, a drug of last resort for therapeutics. In 8–10 days phage treatment by swabbing, lesion sizes showed 7-fold reduction compared to control fish that did not receive phage treatment. The study demonstrated an effective phage treatment against a highly antibiotic resistant *P. aeruginosa* in aquaculture.

ix. Streptococcus iniae

Streptococcus iniae is a Gram-positive, β-hemolytic, zoonotic bacterium that causes streptococcosis in fish as well as endocarditis, meningitis, and arthritis in humans. Streptoccosis has been associated with 30–50% deaths in some fish ponds and is particularly invasive toward tilapia (*Oreochromis* spp.). In Japanese flounder (*Paralichthys olivaceus*) i.p. injection of *S. iniae* and phage cocktail (after 1 h) recorded 50% survival against zero survival in untreated fishes [31]. The delay in phage cocktail injection by 12 and 24 h reduced the survival to 40% and 30%, against none in control. Phage-treated fish that died during the trials often contained phage-resistant *S. iniae*, indicating that further research is needed to establish the effectiveness of phage therapy.

Conclusion

Aquaculture facilities worldwide continue to experience significant economic losses because of diseases caused by pathogenic bacteria. This scenario drives the search for effective biocontrol methods. Phage therapy is currently considered as a viable alternative to antibiotics for control of bacterial pathogens in aquaculture systems. However, several challenges such as mass production of phages and field level efficacy have to be addressed to produce practical, applicable and viable phage therapy technology in aquaculture.

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III. Vaccines and RNAi

Vaccines for viral diseases of fish

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Introduction

World fish production reached 167.2 million tonnes in the year 2014 out of which aquaculture has contributed 73.8 million tonnes [1]. Aquaculture production has peaked in the recent years due to intensification and diversification. The high growth rate in aquaculture also had a price to pay. Intensive culture of fish has resulted in several disease outbreaks resulting in production loss. Among the diseases, viral diseases spread rapidly causing acute mortalities. They are not easily amenable to any treatment measures. Disease management includes establishing good biosecurity protocols, adopting good management practices, early diagnosis of the disease and treatment. Since most disease causing viral agents do not have a specific antiviral drug which can control and treat the infection, prophylaxis is the only reliable method of controlling viral infections, provided an effective vaccine is available.

Viral diseases of cultured fish

Viruses cause 22.6% of the infections in cultured finfishes. However, control of viral infections is difficult due to the rapid spread of infection and unavailability of specific chemotherapeutants. The viral infections of farmed finfish along with the causative agent, clinical signs, hosts affected are given in table 1.

Table 1: Viral disease of farmed finfish

Disease	Host species	Pathogen	Viral morphology	Viral genome
Koi herpesvirus disease	Carps and carp varieties such as Koi carp and ghost carp	Cyprinid herpesvirus 3	Enveloped, Icosahedral symmetry, 100–110 nm in diameter	dsDNA, 277 kbp
Infectious haematopoietic necrosis	Salmons and trout	Infectious haematopoietic necrosis virus	Enveloped, bullet shaped 160 x 90 nm	Non-segmented, negative-sense, single-stranded RNA

Disease	Host species	Pathogen	Viral morphology	Viral genome
Epizootic haematopoietic necrosis	Redfin perch and Rainbow trout	Epizootic haematopoietic necrosis virus	large icosahedral virus (150–180 nm)	linear, dsDNA, 150-170 kbp
Infectious pancreatic necrosis	Rainbow trout, Brook trout, Brown trout, Atlantic salmon	Infectious pancreatic necrosis virus	Non-enveloped, icosahedral measuring about 60 nm in diameter	Bi-segmented dsRNA, segment A (3.1 kbp) and segment B (2.8 kbp)
Lymphocystis disease	Herrings, Smelts, batfishes, killifishes, scorpion fishes, sunfishes etc.	Lymphocystis disease virus-1	Icosahedral virus, approximately 200-300 nm in diameter	Single linear dsDNA of 102.6 kbp
Oncorhynchus masou virus disease	Salmon and Rainbow trout	Salmonid herpesvirus type 2 (SalHV-2)	Icosahedral, enveloped, 200-240 nm	dsDNA
Spring viraemia of carp	Common carp, grass carp, silver carp, bighead carp, crucian carp, goldfish etc.	Spring viremia of carp virus	Bullet-shaped, 80–180 nm in length and 60–90 nm in diameter	Negative sense single stranded linear RNA of ~11 kb
Viral hemorrhagic septicemia	Rainbow trout, turbot, Japanese flounder as well as a broad range of wild freshwater and marine species	Viral hemorrhagic septicemia virus	Bullet shaped measuring about 180 nm long and 60 nm in diameter	12-kb negative sense single-stranded RNA
Viral encephalopathy and retinopathy	Asian sea bass, European sea bass, turbot, halibut, Japanese parrotfish, red-spotted grouper, and striped jack	Nervous necrosis virus	Icosahedral, non-enveloped, 25-30 nm in diameter	Two positive-sense RNA molecules-RNA1 (3.1 kb) and RNA2 (1.4 kb)

Disease	Host species	Pathogen	Viral morphology	Viral genome
Carp pox	Carp and koi carp	Cyprinid herpesvirus-1	Icosahedral, enveloped	dsDNA, 291 kbp
Red sea bream iridoviral disease	Red sea bream and more than 30 other species of cultured marine fish	Red sea bream iridovirus	Non-enveloped, icosahedral, 200–240 nm in diameter	dsDNA, 112 kbp

Vaccines

A vaccine consists of a killed or avirulent or attenuated pathogen as a whole or a part of it which can stimulate the immune system of an animal to produce a specific response and memory. Upon a natural infection the already primed immune system of the animal mounts a prompt response thereby producing specific antibodies against the pathogen resulting in the elimination of the pathogen from the host.

Properties of viral vaccines

Following are the ideal properties of a viral vaccine :

- The vaccine should be safe to the host, the vaccinator and the consumer without causing adverse reactions or vaccine marks in the host.

- The vaccine should produce long lasting immunity, ideally till the production cycle of the fish.

- The vaccine should be able to induce cell mediated, humoral and mucosal immunity.

- The vaccine should be 100% effective against all strains and serotypes of the viral pathogen in a wide variety of hosts.

- The virus present in the vaccine should not regain virulence.

- The vaccine should be easily administrable.

- The vaccine should be economical.

- The vaccine should have a long shelf life.

- The vaccine should not pose ethical issues in licencing.

Immune response to vaccination

The innate immunity comprising of molecules such as interferon are induced in quick response to vaccination. Subsequently, the adaptive immune system of fish comes into play. Immunoglobulin M (IgM) is the major immunoglobulin of fish. The B lymphocytes upon antigen presentation secrete IgM and are found in the serum and mucous of gill, skin and intestines. The secretion of IgM is maximum in case of intraperitoneal vaccination. IgM is also secreted into the mucus when immunised by immersion or by oral routes. In addition to mucosal IgM, systemic IgM is also produced upon immersion and oral vaccination. IgT is reported to be an intestinal immunoglobulin, the equivalent IgA in mammals, and is produced in the intestines upon exposure of mucosa associated lymphoid tissue to antigens [2]. IgD is also a mucosal-immunoglobulin, the transcripts of which are upregulated many fold in immersion vaccinated fish, suggesting that this immunoglobulin plays a major role in mucosal immunity [3].

Types of viral vaccines

There are several types of viral vaccines and new types are being developed continually. The vaccine types commonly available are as follows.

Killed vaccine

Killed vaccines consist of virulent pathogens which have been inactivated by chemicals or by heat. The virus no longer can multiply in the host. They are easy to produce and are economical. The virus is usually inactivated by heat or chemicals or by a combination of both.

Live attenuated vaccine

Live vaccines consist of pathogens which have been rendered incapable of causing an infection or contain an avirulent strain of the pathogen. The virus is attenuated by passaging it in an unnatural host for prolonged time till its infectivity is lost. Live vaccines have the advantage that they can stimulate both cell mediated and humoral immunity. Further the vaccine virus can multiply in the host and result in longer immunity.

Subunit vaccine

Subunit vaccine consists of a portion of the viral pathogen, viz. a particular protein or a peptide, which can induce an immune response in the host against the pathogen.

Recombinant vector vaccines

Recombinant vector vaccines consist of an avirulent virus having antigenic components of a virulent virus. Thus the live virus cannot produce an infection while the

virulent virus portion present in the recombinant virus can trigger an immune response. The vaccine has the advantages of both live vaccine and subunit vaccine.

DNA vaccine

DNA vaccine consists of a plasmid vector containing a portion of the viral genome which codes for some of the immunogenic proteins of the virus. Once administered, the DNA vaccine synthesizes the viral protein using the host cell machinery, thus expressing the viral protein in the host which results in an immune response against the pathogen.

Autogenous vaccine

An autogenous vaccine is usually a killed vaccine prepared using the pathogen isolated from the epizootic for which a licensed vaccine is not available. Autogenous vaccines are prepared to reduce the loss due to diseases till licensed vaccines are made available.

Vaccination methods

The route of vaccine administration greatly influences the immune response of the fish and the protection offered by the vaccine. Injection vaccination which is usually done by intraperitoneal injection offers the best protection in terms of specific humoral antibody production [3]. However, recent research findings suggest that fish has a well-developed mucosal immunity and exposure of mucosal surfaces stimulates the mucosa associated lymphoid tissue (MALT) viz., gut associated lymphoid tissue (GALT), skin associated lymphoid tissue (SALT) and gill associated lymphoid tissue (GIALT) resulting in a mucosal immune response [4, 5]. Immunization routes exposing the MALT produce better protection to fish at mucosal surfaces which are the natural routes of pathogen entry.

Several routes of vaccination have been studied with varying results. The following are some of the routes of immunization used for fish vaccination.

1. Injection vaccination

Injection vaccines usually produce higher systemic immune response in terms of specific antibody production than other routes of immunization. The duration of immunity is also longer probably due to the prolonged release of the antigen when administered along with adjuvants. The size of the fish needs to be 20 g or more for vaccination. However injection vaccination causes handling and injection stress to the fish. To minimize the stress fish needs to be anaesthetized before handling and injecting. Fish can be anaesthetized with tricaine methanesulfonate at a dose rate of 100 mg L^{-1} or clove oil at a dose rate of 50 ppm.

Injection vaccination is labour intensive and also requires trained manpower for injecting the animals. Vaccine can be administered by either manually using an automatic syringe which delivers a predefined volume of vaccine for each stroke of the piston or by using an automated system where the fish are fed in a conveyer belt and fish are vaccinated by automatic vaccinators [6]. A trained person can inject about 1000- 1200 fish an hour [7] while an automated system can vaccinate about 7000 to 9000 fish in an hour [8]. However, automated system is cost intensive and requires relatively larger fish for vaccination. This restricts its use to farmed high value fish such as salmon and rainbow trout which grow to relatively larger size. Vaccination using an automated system is less stressful to fishes compared to manual injection [9].

Vaccination causes release of corticosteroids immediately after vaccination. Increased corticosteroid levels are found to deplete lymphocyte population in circulation and in lymphoid organs and have immunosuppressive effects. However, injection vaccination produces significant immune response which overcomes the stress and immunosuppressive effect.

Injection vaccination can be monovalent or multivalent. Multivalent vaccines provide protection against many diseases simultaneously. However, fishes respond differently to different vaccine components due to the competition between antigens and some antigens may cause non-specific immunosuppression.

Injection vaccination also produces specific memory and booster doses produce higher antibody levels and protection. Primary vaccination induces T-helper cells which are short lived while subsequent booster doses stimulate long lived memory cells and higher antibody levels although the increase do not match the mammalian immune system. Also the increase in the antibody affinity observed in mammals is not observed in fishes.

2. Immersion Vaccination

Immersion vaccination is the simplest route of vaccine administration. The procedure consists of immersing the fish in diluted vaccine solution for a certain period of time. Immersion vaccine can be administered with minimal handling by reducing the water level in the pond/tank or by transferring the fish to a holding tank. A large number of fish can be vaccinated in a short time with minimal labour involvement. However a large quantity of vaccine is required for the procedure.

Immersion vaccination can be administered by many methods viz., Direct immersion (DI), Hyper-osmotic Infiltration (HI), Bath vaccination, Flush vaccination etc.

i. Direct immersion:

In this method, the fish are collected and held in a holding tank containing the vaccine for a particular period of time, say one hour, and then shifted to the culture area. This method is ideal for small fish and is practised before stocking them in the culture ponds.

ii. Hyper-osmotic infiltration:

In this method, the fish are subjected to a hyper-osmotic stress by immersing them briefly in a hypertonic solution like urea or sodium chloride before immersing the fish in the vaccine solution. The vaccine can also be added to the hypertonic solution and the fish may be exposed to the hypertonic solution and vaccine simultaneously. This method is suitable for small fish just before stocking. However, this method causes stress to the fish. Although the increase in immunity and protection offered is variable, in general, hypertonic infiltration gives a better protection to the fish compared to DI [10].

iii. Bath vaccination:

This procedure involves lowering the water level in the holding tank/pond and addition of vaccine to the water. This method can be practised for all sizes of fish. However, large quantities of vaccines are required. This method is less stressful as there is no handling of fish. Higher dilutions of vaccine require longer bath duration for an effective immune response while for lower dilutions shorter bath duration is sufficient. However, studies reveal that higher dilution and longer duration provides better protection than lower dilution and shorter duration [11].

iv. Flush vaccination

Flush vaccination is similar to bath vaccination, except that the water level is not reduced. This method can be followed for all sizes of fish. However, this method is less practised due to the requirement of large volume of vaccine. This method is the least stressful as fish is not handled and there is no change in the fish environment.

v. Spray vaccination

This method involves spraying the vaccine on the fish. The procedure requires less quantity of vaccine and can be practised for larger fishes. This is usually adopted when fish are shifted within the rearing facility. This vaccination method is stressful since the fish has to be netted and carried on a conveyer belt for vaccination [7].

vi. Ultrasound:

This is a technique by which fish are immunised in water containing vaccine and is subjected to high frequency sound waves of about 20 kHz. This enhances the permeability

of the cells and uptake of antigen [12]. This technique requires less concentration of antigen and is said to give protection comparable to injection vaccination [6]. This method is ideal for administering DNA vaccines.

Factors affecting antigen uptake following immersion vaccination

The uptake of antigen following immersion vaccination depends on the nature of the vaccine, concentration of the antigen, pH, osmolality of the water, water temperature and the stress the fish is subjected to. Among these factors higher concentration of the antigen and longer duration appears to offer better protection than vice versa. Further, bigger the size of the vaccinated fish the higher is the immune response. The water temperature also affects the antigen uptake and immune response. The higher the temperature, the higher the antigen uptake and the better the immune response.

The antigen enters the body through the skin, gills and the intestinal tract [13]. The specific serum antibody levels are elevated following vaccination, suggesting that immersion vaccination stimulates skin mucosal tissues [14]. The magnitude of elevation of serum antibody level in immersion vaccination is lower than the antibody response subsequent to injection vaccination [3]. However, the mucosal antibody levels in skin mucus and gill mucus is elevated following immersion vaccination [15]. Studies have demonstrated that the serum and mucosal IgM levels do not always correlate with protection. Recent studies suggest that Ig levels other than IgM, viz., IgT and IgD, appear to be elevated at mucosal surfaces and may account for the protection offered in addition to the involvement of cell mediated immunity [3].

Immunological memory exists in the mucosal immune system and subsequent booster doses result in increased antibody levels and protection.

3. Oral Vaccination

In the oral method, the vaccine is administered usually through feed. The vaccine is either premixed while preparing the feed or coated on the feed usually before feeding [6]. Vaccine can also be administered orally by intubation, a technique by which the vaccine dose is directly administered in the pharynx. However, intubation is used only for experimental trials as it is not practical to administer the vaccine orally to individual fish as it is labour intensive, stressful and may cause mechanical injury in the mouth of the fish [16].

Micro and nano particles can be used to encapsulate or conjugate the antigen. Many micro and nano particles have been experimentally tried and found to be useful for vaccination. Nanoparticles are more efficient in antigen delivery and are more uniform in size. The particles may be natural or synthetic polymers. Particles like alginate, chitosan,

polylactic co-glycolic acid (PGLA) are some of the particles used in oral vaccination. These micro and nanoparticles are also useful in delivering DNA vaccines. Chitosan has many advantages, being a natural polysaccharide obtained from crustaceans is non-toxic and biodegradable. Many researchers have used chitosan nanoparticles for DNA vaccination successfully. Chitosan particles have the added advantage that they have mucoadhesive properties and thus adhere to the skin and gill mucus enabling effective antigen uptake [6].

4. Anal intubation

Oral vaccination has the disadvantage that the antigen is degraded in the acidic environment in the foregut before it reaches the hindgut where the GALT is located. To overcome this, the vaccine can be administered by anal intubation. In this method the vaccine is administered into the hindgut through the anus using a special type of blunt-end syringe or a micropipette. The antigen is taken up by the lymphoid tissue and a local mucosal and systemic immunity is developed. However, this method has the disadvantage that the fish needs to be handled individually and anal administration may cause injury if sufficient care is not taken.

Viral vaccines for farmed finfish

Vaccination is an effective method for control of diseases of farmed finfish. A number of vaccines have been developed and are commercially available for the control of viral diseases. Most of the vaccines available commercially are inactivated vaccines containing formalin or heat killed virus. These vaccines are mostly administered by intramuscular or intraperitoneal injections and hence they are used for bigger fishes as it is practically not possible to inject smaller fishes.

Since many viral diseases appear in the early stage of fish, alternative vaccination methods such as immersion and oral vaccines have been developed. Immersion vaccines are easy to administer, especially for smaller fish in large batches without causing much stress to the fish. However, repeat booster dose administration is a problem.

Oral vaccines are easy to administer to fishes of all sizes and repeated booster doses can be administered easily. The major concerns of oral vaccines are the stability of the antigen during the storage period and degradation of the antigen in the foregut of the fish. This problem can be overcome to certain extent by micro or nano encapsulation of the antigen in particles such as chitosan, alginate etc. More efforts are required to increase the efficacy of oral vaccines, which is a promising method of vaccinating farmed finfish with least stress. Commercially available vaccines for some of the common viral infections are given in table 2.

Table 2: Commercially available viral vaccines for finfish

Name of the product	Type of vaccine	Delivery method	Disease/Pathogen	Target Species	Produced by
AQUAVAC® IPN Oral	Recombinant	Oral vaccine	Infectious pancreatic necrosis (IPN) virus	salmon fry	Merck Animal Health
NORVAX® Compact PD	Inactivated vaccine	Intra-peritoneal injection	Salmonid Alphavirus (SAV)/ Pancreas disease	Atlantic salmon	Merck Animal Health
NORVAX® Minova 6	Inactivated, multivalent vaccine	Intra-peritoneal injection	Furunculosis, classical vibriosis, coldwater vibriosis, wound disease and infectious pancreatic necrosis (IPN)	Atlantic salmon	Merck Animal Health
KV3 Vaccine	Attenuated virus vaccine	Immersion in a tank and Injection	KHV disease	Common Carp and Koi carp	KoVax Ltd., Israel
ALPHA JECT micro® 6	Inactivated, multivalent	Intra-peritoneal injection	*Aeromonas salmonicida, Vibrio salmonicida, Listonella anguillarum, Moritella viscosa* and IPN	Atlantic salmon	PHARMAQ AS, Norway
ALPHA JECT® 2-2	Inactivated	Intra-peritoneal injection	Furunculosis, IPN	Atlantic salmon	PHARMAQ AS, Norway
Autogenous VNN vaccine	Inactivated	Intra-peritoneal injection	Viral Nervous Necrosis	European Sea bass	PHARMAQ AS, Norway
ALPHA JECT MICRO 1 PD	Inactivated	Intra-peritoneal injection	Salmon Pancreas Disease Virus (SPDV)	Atlantic salmon	HPRA, Ireland

Name of the product	Type of vaccine	Delivery method	Disease/Pathogen	Target Species	Produced by
ALPHA JECT® micro 1 ISA	Inactivated	Intra-peritoneal injection	Infectious salmon anaemia	Atlantic salmon	PHAR-MAQ AS, Norway
Subunit vaccine against ISA oral powder	Subunit vaccine	Oral	Infectious salmon anaemia	Atlantic salmon pre-smolt, from 10 g of body-weight.	Virbac

Concluding remarks and future outlook

Vaccination of finfish has a clear advantage of reducing the impact and loss due to diseases, reducing the use of chemotherapeutants and provide long-term protection. Hence vaccination of fish is an important tool in health management. At present most of the vaccines available commercially are for salmonids which account for 6.2% of the total aquaculture production. However, cyprinids contribute about 62.7% to the total production. Hence there is a huge potential for the development of vaccines for carps and other cyprinids. Even if vaccination can marginally improve the survivability of fish, it can offset the vaccine and vaccination costs. Optimization of vaccine dose for protection of fish, development of anti-fish antibody for seromonitoring of vaccinated fish and establishment of protective antibody titre required to withstand a natural infection are areas for immediate research in the field of fish vaccinology.

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Bacterial Vaccines for Fishes: Current Status

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Bacterial Diseases are a major constraint to aquaculture production globally, impeding economic and social development in many countries. In the USA alone, the annual loss due to only two bacterial pathogens i.e. *Flavobacterium columnare* and *Edwardsiella ictaluri* is estimated to be $ 50–70 million [1]. Similar situation exists in several other countries of the world. A total of 92 bacterial genera have been reported to be pathogenic for fish [2]. The majority of bacterial pathogens of fish belong to the families *Enterobacteriaceae, Aeromonadaceae, Psuedomonadaceae, Vibrionaceae, Flavobacteriaceae* and *Streptococcaceae*.

Although bacterial diseases can be treated with antibiotics, the emergence of multi-resistant bacterial pathogens not only constitutes a potential threat to public health but also renders antibiotic treatment increasingly difficult. Compared to antibiotics, vaccines have the advantage of being safe, environmentally friendly, and offering long-term protection. In general, there are two major classes of bacterial vaccines: (1) Replicative vaccines (2) Non-replicative vaccines.

Replicative vaccines - These vaccines include live attenuated pathogen that replicates inside the host. Attenuation strategies used to develop live vaccines for fish include antigen mimicry, laboratory passage, chemical or physical mutagenesis and genetic modification using molecular techniques. Advantages: (1) Infectious microbes can stimulate generation of memory of cellular as well as humoral immune responses. (2) Since they can multiply in the host, fewer quantities are required to induce protection. (3) A single administration of vaccine often has a high efficacy in producing long-lived immunity. (4) Whole microbes stimulate response to antigens in their natural conformation. They raise immune response to all protective antigens. (5) Some live vaccines can be given orally; such vaccines induce mucosal immunity and IgA synthesis, which gives more protection at the normal site of entry. Disadvantages: (1) May very rarely revert to its virulent form and cause disease. (2) Since they are live and because their activity depends on their viability, proper storage is critical. (3) Live vaccines cannot be given safely to immunosuppressed individuals.

Non-replicative vaccines - These vaccines include an inactivated pathogen that does not replicate inside the host called *inactivated/killed vaccines*. Another category is s*ubunit vaccines*- Subunit vaccines are the specific immunogenic proteins of the bacteria purified from pathogen or expressed in a recombinant vector.

Inactivated or killed vaccines consist of bacteria or other pathogens that have been cultivated in artificial culture and for use as vaccine agent, killed using physic-chemical methods. Killing or inactivation of the live cells in such vaccines is performed as a measure to reduce infectivity (virulence) and thus preventing infection from the vaccine. They have several advantages such as easy to produce, eco-safe and low cost. Their storage requirements are not as critical as live vaccines. However, they have some disadvantages: (1) Since the bacteria cannot multiply, a large number are required to stimulate immunity, (2) Periodic boosters must be given to maintain immunity, (3) Only humoral immunity can be induced, (4) Inactivation may alter antigenicity and (5) Presence of some un-inactivated microbes can lead to vaccine-associated disease.

Subunit vaccines use specific epitopes of the antigen that bind to antibodies or T cells. Because subunit vaccines contain only the essential antigens and not all the other molecules that make up the microbe, the chances of adverse reactions to the vaccine are lower. Subunit vaccines are produced in large quantities either from organism directly or *in vitro* as recombinant proteins through different eukaryotic and prokaryotic expression. The advantages are (1) they can be safely given to immunosuppressed animals (2) less likely to induce side effects. Disadvantages: (1) Antigens may not retain their native conformation, so that antibodies produced against the subunit may not recognize the same protein on the pathogen surface. (2) Isolated protein does not stimulate the immune system as efficiently as a whole organism.

DNA vaccines rely upon the use of genes encoding protective antigens, instead of the antigens themselves. These are genetic constructs containing one or more genes obtained from a pathogen, and they are designed to facilitate the transient production of protein from these genes in the vaccine with the aim to elicit a protective immune response. DNA vaccines are usually in the form of a purified bacterial plasmid. The constructs contain regulatory elements to ensure both replication of the plasmid in the bacterial host for production purposes, and expression of the genes in the vaccinated animals. Advantages: (1) DNA is very stable, it resists extreme temperature and hence storage and transport are easy. (2) A DNA sequence can be changed easily in the laboratory. (3) The inserted DNA does not replicate and encodes only the proteins of interest. (4) There is no protein component and so there will be no immune response against the vector itself. (5) Because of the way the antigen is presented, there is a cell-mediated response that may be directed against any antigen in the pathogen. Disadvantages: (1) Potential integration of DNA into host genome leading to insertional mutagenesis. (2) Induction of autoimmune responses: anti-DNA antibodies may be produced against introduced DNA. (3) Induction of immunologic tolerance: the expression of the antigen in the host may lead to specific non-responsiveness to that antigen.

Status of Vaccine Development for Major Bacterial Diseases of Fish

Vibriosis: Infections caused by *Vibrio* species mainly *Vibrio anguillarum*, *V. alginolyticus*

Inactivated vaccines

Immersion: A licensed bacterin (GAVA-3) covers O1, O2a and O2b highly pathogenic serotypes of Vibrio [3]. The majority of commercial vaccines available appears to be mainly for serotypes O1 or O1+O2a (MicroVib, Alpha-Marine, Alpha Dip and Aqua-Vac *Vibrio*[4]. A formalin inactivated trivalent vaccine for sero-subgroups O2a, O2b and O2c was tested on juvenile Atlantic cod. The vaccine resulted in efficient protection against all sero-subgroups [5].

Live vaccine

Immersion: Two live attenuated vaccines constructed by transposon insertion mutagenesis and one antibiotic-resistant mutant has been demonstrated to provide immunization against a homologous as well as a heterologous strain of *V. anguillarum* for at least two weeks in rainbow trout [6].

Intra-peritoneal: A live recombinant vaccine secreting DegQ soluble antigen, against *V. harveyi* in turbot has been shown to be effective by IP, oral and immersion [7].

DNA vaccine

Intra-peritoneal: A plasmid DNA vaccine construct containing flagellin *flaA* gene has shown 88% RPS[8]. Likewise a bivalent *V. harveyi* DNA vaccine expressing DegQ and Vhp1 immunogens has been tested in Japanese flounder with more than 70% RPS[9]. This vaccine also elicited cross protection against *V. parahaemolyticus.* DNA vaccine expressing ompW has 92% RPS [10]. Recently, a recombinant polyvalent DNA vaccine containing OmpAs from different bacterial pathogen has been developed using DNA shuffling approach. This vaccine was able to immunize fish against *V. alginolyticus* and *E. tarda* infections [11].

Intra-muscular: DNA vaccine containing *omp*38 was administered intra-muscularly into the Asian seabass, showed significant rise in serum antibody levels against *V. alginolyticus* with 55.56% of relative percent survival (RPS) [12].

*Oral: omp*38 DNA vaccine when administered orally along with chitosan nanoparticles in Asian seabass gave relatively lower RPS (46%) [13]. Similarly, another omp based DNA vaccine, containing *ompK,* was evaluated in black seabream against *V. parahaemolyticus* infection. When the vaccine encapsulated in chitosan was fed orally, it evoked immune response within 3 weeks with a survival rate of 72.03% [14].

Subunit vaccine

Out of four flagellins (rlaA, rflaB, rflaC, rflaD, and rflaE) of *V.anguillarum*, rflaB vaccine gave higher level RPS in flounder by IP injection [15].

Motile Aeromonads septicaemia disease: Infection by *Aeromonas hydrophila*

Inactivated vaccines

Oral: Biofilms vaccines appears to have effective responses in many fish species [16-18]. These vaccines elicited the high serum antibody and protective response in three carp species up to 60 days [17].

Intra-peritoneal: IP administration of biofilm, extracellular product, *omp* and whole cell vaccines also have good response in goldfish. Immunization with biofilm and *omp* along with immunoadjuvant *Asparagus racemosus* tuber powders showed significantly increased survival after 25 and 50 days post vaccination[19]. A formalin killed whole cell vaccine showed RPS of 80% [20]. In rainbow trout, IP administration of bacterial lysate significantly lowered mortality [21].

Live vaccine

Immersion: Patented live attenuated vaccine strains of *A. hydrophila* were developed through induction of rifampicin resistance. It showed 100% RPS against virulent *A. hydrophila* infection in fish in single immersion exposure [22].

Intra-peritoneal: Transposon mini-Tn5 mutagenesis-induced growth-deficient mutants used as vaccine resulted in 40% increase in survival [23]. An aroA gene mutated strain conferred significant protection [24]. Effective immunization of *Labeo rohita* with rough lipopolysaccharides containing live attenuated vaccine having 100,000-fold less virulence, developed by Swain et al. [25], after simple laboratory passage for 8 years has been observed. A Novobiocin and rifampicin-resistant live strain demonstrated 86-100% protection in channel catfish and Nile tilapia using IP injection [26].

Subunit vaccine

Oral: Live recombinant *Lactococcus lactis* vaccines expressing aerolysin genes provided high RPS [27]. A dose dependent protective immunity in rohu was observed when a recombinant outer membrane protein OmpW was orally administered along with PLGA nanoparticles [28].

Intra-peritoneal: Recombinant rOmpR demonstrated significantly reduced mortality 140 days post immunization [29]. Outer membrane protein ompTS based recombinant vaccine immunization of Indian major carp has demonstrated high antibody titres on

day 28 post vaccination [30]. Bath and intramuscular injection immunization by Single-walled carbon nanotubes-aerA subunit vaccine has shown 80% of survival in vaccinated fish [28].

Furunculosis: Infection by *Aeromonas salmonicida* subsp. *salmonicida*

Killed vaccine

Intra-peritoneal: Low frequency sonophoresis (LFS) adjuvanted bacterin was intra-peritoneally given in rainbow trout which resulted in high up regulation of IgM antibody [31].

Live vaccine

Immersion: Immersion vaccination of rainbow trout with attenuated vaccines containing deficiency of A-protein, O-antigen or both was performed which resulted in significant protection by all strains from challenge with a heterologous virulent strain of *A. salmonicida* [32].

Intra-peritoneal: A live attenuated *aro*A deficient mutant was used to immunize brown trout with significant protection against live infection [33]. Another live attenuated vaccine developed from *aro*A deficient mutant of *A. salmonicida* was demonstrated with more than 60% RPS in rainbow trout [34]. Romstad et al. [35] evaluated RPS of Atlantic salmons intra-peritoneally immunized with 10 commercially available vaccines. They demonstrated RPS at LD60 was maximum in comparison with RPS at LD90. Mineral oil-adjuvanted two vaccines; AlphaJect 3000® and an experimental auto vaccine were tested on rainbow trout and 78% and 56% RPS was demonstrated respectively [36].

Enteric Septicemia of Catfish: Infection caused by *Edwardsiella ictaluri*

Live vaccine

Immersion: A USDA approved *E. ictaluri* strain RE-33 live vaccine has been shown to provide immunization for at least 4 months following a single bath immersion without any risk hazard [37] and later the same vaccine strain was patented [38]. Modified version of this vaccine, AQUAVAC-ESC™ is being marketed with 87.9% RPS [39]. A novel attenuated *E. ictaluri* vaccine agent (B-50348), developed through selection for novobiocin resistance mutant, was successfully tested for immersion and intra-peritoneal vaccination [40]. Live vaccine containing a cyclic adenosine 3',5'-monophosphate receptor protein (CRP) mutant demonstrated high IgM titers after bath immunization in catfish [41].

Intra-peritoneal: aroA attenuated vaccine strains against *E. ictaluri* were produced and demonstrated to be effective in laboratory studies [42]. Transposon mutagenesis was used

to generate an O polysaccharide-deficient isolate of *E. ictaluri* for use as a live vaccine, but without protection [43].

Oral: Oral immunization with rifampicin resistant live vaccine (S97-773) has offered 82.6-100% RPS [44].

Subunit vaccine:

Mutants carrying double gene mutations in TCA cycle and C1 metabolism were developed and evaluated for vaccination by immersion. Fish vaccinated with mutants demonstrated 100% survival [45].

Edwardsiellosis: Infection by *Edwardsiella tarda*

Killed vaccine

Intra-peritoneal: Formalin-killed *E. ictaluri* whole cells and an *E. ictaluri* rGAPDH combined vaccination adjuvanted with ISA 763A in tilapia showed 71.4% RPS post 3 month immunization on challenge with virulent *E. tarda* [46]. A killed, but metabolically active (KBMA) *E. tarda* vaccine in olive flounder showed significantly higher survival rates than fish immunized with formalin-killed cells [47].

Live vaccine

Intra-peritoneal: A live attenuated vaccine, with mutation in aroC gene was evaluated in turbot showed long protection [48]. Booster immunization of a temperature-sensitive *E. tarda* mutant induced 100% protection [49]. A recombinant live *E. tarda* mutant, lacking UDP-glucose dehydrogenase showed 76.7% RPS [50].

Subunit vaccine

Intra-peritoneal: Recombinant *rFimA* elicited high level of protection in turbot [51]. A recombinant vaccine *rGAPDH* significantly increased transcription levels of immune genes of vaccinated fish [52]. Recombinant *FlgD* protein was used to immunize a zebrafish model and RPS of about 70% was observed [53].

DNA vaccine

DNA vaccine encoding molecular chaperone *GroEL* showed an RPS of 60% [54].

Columnaris disease: Infection by *Flavobacterium columnare*

Inactivated vaccines

Bath: Bath immunization with a bacterin has shown to protect carp against experimental challenge, but no antibodies were detected in sera of immunized fish [55]. In channel

catfish, vaccination by immersion in a bacterin has shown significant decrease in mortality compared to unvaccinated fish [56]. Bacterin-based immersion vaccine (Fryvacc 1) is available for use in salmonids in the US and Canada. Fryvacc 2 is a bivalent vaccine containing *F. columnare* and *Yersinia ruckeri* bacterins, and is available in Chile [57]. Coating bacterins in alginate microparticles did not improve the efficacy of vaccine in tilapia [58].

Oral: Protection in coho salmon has been demonstrated with heat-killed cells of *F. columnare* incorporated into fish feed [59]. Similarly, feeding for over three months of formalin-killed bacteria provided high levels of protection [60].

Intra-peritoneal: Use of formalin-killed sonicated cells in Freund's complete adjuvant in tilapia resulted in a significant systemic humoral response within two weeks and antibody levels almost tripled following secondary immunization. High antibody levels were observed even at 10 weeks post-immunization [61].

Live vaccine

Immersion: An attenuated immersion vaccine (Aquavac-Col) currently is registered for use in channel catfish fry in USA. Relative percent survival (RPS) ranged between 57 and 94% after challenge with virulent *F. columnare*. In largemouth bass fry, RPS values were between 74 and 94%, depending on the vaccine dose [62]. A rifampicin-modified *F. columnare* has been developed and patented by USDA-Agricultural Research Service. The efficacy of vaccine administered singly or with a booster vaccination was shown to be protective with RPS values ranging from 50.0 to 76.8%.

Oral: Vaccinated largemouth bass fry showed 43% lower risk of death by *F. columnare* during the field trial [63].

Bacterial cold-water disease: Infection by *Flavobacterium psychrophilum*

Bacterial cold-water disease is caused by *F. psychrophilum*, and is known to affect a variety of cold-water fish species.

Inactivated vaccines

Positive correlation is seen between specific antibody response and protection against *F. psychrophilum* in rainbow trout [64-66] and in ayu [67, 68] after IP immunization, but not with immersion [64, 69]. Oral vaccinations of ayu [70] and rainbow trout [71] have also been successful.

Subunit vaccines

Low protection is seen with subunit vaccines without the use of conventional adjuvants [66, 67, 72] except IP immunization of rainbow trout fry with a recombinant protein combined with an adjuvant [73]. IP immunization of rainbow trout with a 70–100kDa fraction in combination with an adjuvant resulted in high specific antibody titers with high survival rates [74]. Recombinant heat-shock proteins/high molecular weight proteins or DNA vaccine failed to confer protection [72, 75]. IP immunization of outer membrane proteins are reported to be protective [67, 76, 77].

Live vaccines

Several types of live attenuated strains (growth under iron-limited conditions [78]), (rifampicin resistant [79]) have been used for vaccination trials with high to moderate success.

Photobacteriosis-Infection by *Photobacterium damselae* subsp. *piscicida*

Photobacteriosis is a bacterial septicaemia, also called Pasteurellosis or Pseudotuberculosis. It affects white perch, striped bass, yellowtail, sea bream, sea bass and sole.

Inactivated vaccines

Most of the commercial vaccines are inactivated using heat or formalin [80]. efficacy of inactivated vaccine delivered by immersion can be improved by the use of ultrasound treatment[81]. Incorporation of oil adjuvants offer better efficacy for injectable preparations [82]. Use of glucose and/or salt-enriched media to grow the bacterial cells [83], and iron-depleted media has shown to offer better protection [84]. Bacterins prepared from extracellular products (ECP) or LPS [85] and capsular polysaccharide [86] also confer higher protection. A formalin-killed bacterin containing over-expressing protective protein has been commercialized by Aqua Health, Canada, under the brand name "Photogen" for use in sea bass and yellowtail [87].

Live vaccines

A siderophore-deficient strain has been used as live vaccine [88]. A live vaccine using aro-A-deletion mutant has been patented in the US for use in hybrid striped bass [89].

Yersiniosis: Infection by *Yersinia ruckeri*

Yersiniosis or Enteric redmouth disease (ERM) is a disease caused by *Y. ruckeri*, which mainly affects young salmonids in hatchery. Immersion-based inactivated bacterins are available for protection against Hagerman' O1 biotype 1 and biotype 2 variants of

Y. ruckeri [90. 91]. The role of antibody-mediated protection of bacterins is not clearly established as *Y. ruckeri* resides within macrophages [92].

Inactivated/subunit vaccines

Injection: The O-antigen of the lipopolysaccharide confers high levels of protection against yersiniosis in trouts [93]. Toxoid of the Yrp1 protease is also known for its protective ability against yersiniosis [94].

Live vaccines

A live vaccine using aro-A-deletion mutant has shown to provide superior protection to the bacterin-based vaccines [95].

Streptococcosis: Infection by *Streptococcus agalactiae*

Streptococcosis is a systemic disease of both cultured and wild fish species. The causative agents are *S. agalactiae,* and *S. iniae*. Currently, AQUAVAC® Strep, an inactivated oil-adjuvant vaccine is commercially available in Brazil that provides protection against *S. agalactiae* infections in tilapia of more than 15 grams by injection.

Inactivated/subunit vaccines

Formalin-killed cells along with concentrated extracellular antigens have been reported to offer significant protection to larger fish, with a RPS of 80% at 30 days post-vaccination [96]. Chen et al. [97] identified 10 distinct pulsed-field gel electrophoresis (PFGE) genotypes (A–J) of *S. agalactiae* and used them to develop an inactivated whole-cell bacterial vaccine.

Live vaccine

A polyvalent live vaccine consisting of 30 isolates of sparfloxacin-resistant *S. agalactiae* was reported to provide significant protection to Nile tilapia against challenge with *S. agalactiae* [98].

Streptococcosis: Infection by *Streptococcus iniae*

S. iniae is an important fish pathogen of tilapia. A bacterin vaccine is currently available in Asia to protect tilapia from *S. iniae* infection [99].

Inactivated/subunit vaccines

A number of bacterins have been tested successfully for protection [100-102]. Some studies show that these bacterins are unable to protect fish from infection by different serotypes of *S. iniae* [103, 104]. Preparations containing both formalin-killed cells and ECP have been reported to partially protect Nile tilapia from infection [105-108].

Live vaccine

Live attenuated strains defective in phosphoglucomutase and M-like protein have been reported to offer protection against homologous *S. iniae* challenge [109,110]. An attenuated novobiocin-resistant strain (named ISNO) has been reported to protect tilapia for at least 6 months [111].

DNA vaccine

DNA vaccine containing putative secretory antigen was reported to offer protection to turbot under laboratory conditions [112].

Lactococcosis: Infection by *Lactococcus garvieae*

Lactococcosis is a systemic disease of both cultured and wild fish species. The causative agent is *L. garvieae*. Commercial vaccines are available for rainbow trout in Italy, France, and UK; and for yellowtail in Japan [99].

Inactivated vaccines

Injectible formalin-inactivated vaccine showed protection rates of 70–80% for three months to trout [101] and yellowtails [113]. Non-mineral Oil-adjuvanted vaccine (Aquamun) offer significantly higher protection (92%) as compared to non-adjuvanted vaccine (40%) at three months after vaccination [114]. Similarly, when formalin-inactivated *L. garvieae* bacterin vaccines were combined with Freund's incomplete adjuvant, the vaccines were found to provide longer protection against virulent *L. garvieae* infections in rainbow trout compared with that without the adjuvant [115].

Live vaccine

A live vaccine using a strain lacking a virulence-associated capsule has shown to provide long lasting protection to yellowtail [116].

Piscirickettsiosis: Infection by *Piscirickettsia salmonis*

Piscirickettsiosis is a septicemia caused by *P. salmonis,* a facultative gram-negative bacterium and mainly affects Chilean salmon.

Inactivated vaccines

A number of bacterins with variable protection have been tested including heat [117] or formalin-inactivated bacterins [118, 119]. Birkbeck et al., [120] proved that high antigen concentrations are essential for protection. Commercial bacterins including oral formulation are available in Chile for the protection of salmon [121, 122].

Subunit vaccines

Recombinant OspA gave high protection in IP challenge [73]. Heat-shock proteins, and flagellin have also been tested as vaccine candidate [123, 124].

Live vaccine

Live vaccine **"**Renogen" developed to control BKD shows significant reduction of mortality due to piscirickettsiosis under farm conditions [125].

Bacterial kidney disease: Infection by *Renibacterium salmoninarum*

Inactivated/sub-unit vaccines

Studies have shown that whole cell bacterins offer variable protection to BKD [126, 127]. The highly abundant, 57kDa extracellular major soluble antigen (MSA or p57 protein) plays an important role in pathogenesis and eliciting immune response. However, retaining of MSA on killed cells confers little or no protection to Pacific salmon through either oral or IP route [128 – 131]. Lowering the levels of MSA in vaccine preparation by either heat treatment [130, 132] or using low producing strains of MSA [133] offers protective immunogenicity without the risk of disease or mortality.

Live vaccines

Live attenuated strains with reduced or normal cell-associated MSA have been tested as vaccines [133 – 135], with little or no protection. Significantly, *Arthrobacter davidanieli*, avirulent bacterium whose surface carbohydrate resembles *R. salmoninarum* has been tested as vaccine candidate. It provides significant protection in Atlantic salmon [125, 134, 136] and is marketed as "Renogen" in several countries.

Conclusions

Presently, for increasing aquaculture production, new fish species are being brought into culture, and as a result new strains or variants of bacterial pathogens are likely to appear. So preventing bacterial disease outbreaks would continue to remain a challenge due to lack of vaccines and vaccination procedure in India. There is a lot of opportunity to develop effective vaccines for bacterial diseases of fish, especially with regard to the use of local bacterial strains and effective mode of delivery.

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Vaccines for finfish parasites

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Introduction

Finfish species from marine, brackishwater and freshwater constitute the bulk of aquatic products and provide nutrition to around 7.3 billion people the world over [1]. As the capture fisheries is becoming stagnant and wild stocks are declining, aquaculture in both marine and freshwater is becoming the major stay of finfish production. To meet the ever-increasing global demand, aquaculture is gradually switching to more intensive than extensive farming and thus putting the fish to more hostile environment. This increases the risk of contacting several infectious diseases of bacterial, viral and parasitic origins. These pathogens, particularly of bacterial/viral origins, are controlled either by therapeutic measures or preventive measures by vaccination. Vaccination to prevent bacterial or viral diseases is attractive owing to its capacity to protect large number of animals for a longer duration. Therapeutic measures are applied only after the occurrence of the diseases and the protection is short-term, i.e., the chances of recurrence of the diseases are always present. Vaccines against a number of bacterial or viral diseases of finfishes are commercially available, e.g. furunculosis vaccine, vibrio vaccines, KHV vaccine, infectious salmon anemia vaccine, etc., which have drastically reduced the loss incurred by these diseases.

Parasitic diseases and their control

Finfishes are also affected by a wide range of parasites belonging to several genera. Some of the important diseases they cause are white spot disease, amoebic gill disease, sea lice infestations etc. leading to heavy economic loss. Besides, there are also other parasitic infestations of lesser importance adding to the loss in finfish production. A conservative estimate of loss due to parasitic infestations in production has been estimated at US$1.05 billion to $9.58 billion per annum [2].

The parasites normally do not induce potent and protective immune responses. Hence, the major method adopted for control of parasitic diseases is chemical treatment. However, the treatment protects the fish only for a short period. Besides, the parasites may develop resistance to these chemicals in addition to its harmful effects on non-target organisms and may bioaccumulate in fish, making it harmful for human consumption. Alternative methods like biological control, control through application of immunostimulants etc. have been attempted against some parasites with limited success [3].

Vaccines against parasites

Vaccination against fish parasites seems to be a safe and effective alternative to the traditional chemical methods, considering its success in several terrestrial animals. There are several vaccines currently available against animal parasites, though not a single vaccine against any parasitic diseases is available commercially for any finfish species. Finfish species in general possess the capability to produce adaptive immune responses to parasitic infections [4,5] and a number of reviews are available even on specific groups of parasites such as flagellates [6], microsporidia [7], ciliates [8], myxozoans [9,10], gastrointestinal microparasites [11] and intestinal helminths [12]. Further, the observations that parasitic infections confer some kind of immunity to subsequent infections by the same parasite, strengthen the possibility of a vaccine strategy. Developing vaccine against parasites in general is a bit tricky, as unlike bacterial or viral pathogens, they contain a wide variety of antigens and also use several immune evasion strategies such as antigenic variation, molecular mimicry etc. Additionally, they exhibit complex lifecycles and other biological characteristics, which complicate the vaccine development process. Thus, the identification of important protective antigen as the vaccine candidate and an appropriate challenge model are important for the successful development of a vaccine against parasites. Researchers the world over are trying to develop vaccines against several important parasitic infections, which are detailed below. Most of the vaccine development processes against the parasites are in experimental stages and in a majority of the cases the development of humoral antibodies and a successful protection against challenge experiment are taken as the criteria for success.

Icthyophthirius multifilis and Cryptocaryon irritans

I. multifilis is a ciliated protozoan affecting the skin and gill of freshwater fishes causing a disease commonly known as Ich or White Spot Disease, resulting in heavy economic loss. Extensive research on vaccines against this parasite has been undertaken; though vaccine development has been difficult owing to the complex life cycle of the species. Clark and Dickerson [13] showed that channel catfish antibodies bind to specific coat protein (glycosylphosphatidyl-inositol-anchored proteins referred to as immobilization antigens, iAg) forcing the parasites to exit the skin prematurely. They later showed the successful immunization of catfish that showed 72% protection, when iAg was used as a subunit vaccine [14]. Different serotypes of iAg are prevalent in nature [15] and it elicits serotype-specific protective response [16]. Vaccine studies have also been conducted with live (theronts) and killed (sonicated trophonts) ciliates, and live ciliates provided better protection, in channel catfish [17] and Nile tilapia [18]. Live theronts, however, provided cross-serotype protection, indicating involvement of other antigens besides iAg [19]. Jørgensen *et al*. [20] tested 3 DNA vaccines encoding two

iAgs and one cysteine protease of *I. multifilis* in *Oncorhynchus mykiss*. The vaccines did not give significant protection even though there was detectable antigen expression and immune reactions and they suggested that additional parasite antigens are required for such a vaccine to be successful. In a recent study, Xu *et al.* [21] showed a 95% protection in channel catfish immunized with live theronts of *I. multifilis*.

C. irritans is another ciliated protozoan that causes Ich in marine fish. Immunization studies using live theronts have been conducted in grouper, *Epinephelus coloides* that showed protection [22]. *Bai et al.* [23] conducted a comparative study of theronts, tomonts and trophonts stages of *C. irritans* in grouper and found theront stage to induce a stronger protective response. An immobilization antigen (iAg) was also identified in *C. irritans* and cloned [24]. Josepriya *et al.* [25] used codon optimization in iAg of *C. irritans* that showed expression in both prokaryotic and eukaryotic cell systems and also showed protection against *C. irritans* infection in grouper. They further enhanced the protective capability of iAg by adjuvanting with the parasitic heat shock protein 70C (Hsp70C) encapsulated together in chitosan nanoparticles [26].

Cryptobia salmositica

The haemoflagellate *C. salmositica* causes a disease salmonid cryptobiosis which is normally transmitted by a freshwater leech, *Piscicola salmositica*. Amongst flagellates, this species has been studied extensively for vaccine development. Since the organism can be cultured in artificial media, the pathogen has been attenuated by repeated subculturing [27]. This strain has been used routinely as vaccine in many experiments and has shown protection in salmonids [27]. Both antibody mediated [28] and cell mediated [29] immune response have been shown to be involved in the protection of vaccinated fish. Later on, a DNA vaccine has been prepared incorporating the metalloprotease gene of the parasite. The DNA-vaccinated fish (*O. mykiss* and *S. salar*) when challenged with the pathogen had consistently lower parasitemia, delayed peak parasitemia, and faster recovery compared with the controls and showed promise as a vaccine candidate (30).

Neoparamoeba perurans

The free-living amoeba *N. perurans* is the causative agent of Amoebic Gill Disease affecting salmonids the world over. Experimental vaccines have been prepared with killed and live amoebae and tested in *S. salar* against this parasite [31]. Cook *et al.* [32] used a DNA based vaccine against this parasite. However, all these vaccines have failed to produce a desired level of protection against this parasite.

Loma salmonae

Microsporidia are obligate intracellular parasites affecting a wide range of fishes in both seawater and freshwater. They produce infective spores that transmit the disease.

Of the many species, *L. salmonae* that causes a disease, microsporidial gill disease of salmon, has been studied in detail towards vaccine development. Live spores from a low virulent strain of *L. salmonae* has been used to develop a vaccine and was found effective in rainbow trout [33]. Involvement of a strong cell mediated immune response in protection with the spore vaccine has been proposed [34]. Speare *et al*. [35] found the intraperitoneal route of vaccination to be effective, and there was no improvement in vaccine performance with addition of adjuvant. The successful vaccination with *L. salmonae* shows promise for development of vaccines in other microsporidial diseases of fish.

Sea lice

Sea lice are ectoparasitic copepods belonging to *Lepeophtheirus salmonis* and *Caligus* sp. that affect primarily salmonids and cause heavy economic loss to the industry [36]. Decades of vaccine research against these parasites involving huge money has not yielded much success possibly owing to the non-availability of proper vaccine antigens to induce protective immunity.

Unlike endoparasites, development of vaccines against ectoparasites is difficult, since the parasite does not enter the host to induce an active immune response to protect themselves. A different approach has been utilized by Wiladsen *et al*. [37] to develop vaccine against cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*. They found a protective protein, Bm86, a concealed antigen of tick gut. The immunized cattle would develop antibody against the protein and enter the tick gut through blood feeding by tick. The antibodies would bind to the tick gut and increase the cell permeability and the tick would die. This approach has been used in several other parasites of terrestrial animals and also employed in developing vaccines against sea lice infestations of salmon.

The initial research on sea lice species has started with crude antigenic preparations from the parasites [38]. Later on, researchers tried to target the gut antigens of the parasite as vaccine candidates. A trypsin-like enzyme was isolated, characterized and used as vaccine [39]. Also antigens, of the reproductive system of the parasites, too, formed the basis of several vaccine researches [40]. Later, the development of genomic information of these species have accelerated the development of various other antigens. Carpio *et al*. [41] characterized a novel gene (denoted as my32) from *C. rogercresseyi*, which has the highest identity with the *L. salmonis* gene akirin-2. They used recombinant my32 protein as vaccine in *S. salar* and challenge experiment showed a 57% inhibition of infestation in vaccinated group. They later studied different strategies to improve vaccination response such as fusing my32 to another physiologically relevant antigen and the use of endogenous

molecules as molecular adjuvants [42]. Recently, a sea lice vaccine against *Caligus* has been launched in Chile in November, 2015 [43].

Compared to sea lice, freshwater fish lice belonging to genus *Argulus* have received much less attention over the years. Ruane *et al.* [44] demonstrated an antibody response in rainbow trout *O. mykiss* after they were immunized with an antigen extract from *A. foliaceus* and suggested the possibility of a vaccine against this parasite. In India, vaccine research has just been initiated against the most prevalent sp., *A. siamensis* [45]. In a project funded by ICAR (National Fund for Basic, Strategic & Frontier Application of Research in Agriculture), an experiment has been conducted with crude antigenic preparations of the parasite resulting in limited protection [46]. Kar *et al.* [47] has also attempted a peptide antigen from ribosomal protein P0 of this parasite to vaccinate rohu (*Labeo rohita*) but could demonstrate only a delayed mortality in vaccinated group. They suggested further optimization in formulation and immunization schedule in utilizing this antigen as a vaccine candidate.

Besides the above parasites, very limited research on vaccines has been undertaken in other parasites of finfish species such as *Amyloodinium ocellatum* [48]; *Discocotyle sagittata* [49]; *Diplostomum pathaceum* [50], etc.

Conclusion

Anti-parasitic vaccines have been attempted against several important parasitic diseases of finfish species with limited success. These attempts suffer from the disadvantages that there still exist gaps in the knowledge of numerous immune mechanisms as well as the host parasite interactions. Besides, identification of protective antigens is most crucial in developing any vaccine against parasites. With the use of modern genomics and proteomics tools, it may now be easier to identify such protective antigens. The successful development of a vaccine against a metazoan parasite (sea lice) definitely show the way for further research in this line and we can expect the availability of a number of vaccines against fish parasites in the near future.

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Recombinant and subunit vaccines for aquaculture

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Introduction

There is increased intensification in the aquaculture industry to meet the growing demand for fish and shellfish in the global market. With increasing intensification and trade of aquacultured animals and their products, many foreign pathogens get introduced into the culture systems, causing disease epizootics. The disadvantages in the use of chemotherapeutics, including antibiotics, are being felt widely. In many developing and under-developed countries, antibiotics are the most reliable control method so far. However, in the recent past, the industry has been facing problems related to antibiotic use such as antibiotic resistance, residual effect, effect to pond indigenous micro flora and fauna, accumulation of antibiotics in fish and shrimp body, etc. "Prevention is always better than cure", so it is always better that we make our fishes more healthy by using different preventive measures to fight different pathogens. One of the effective long-acting methods is the use of vaccines for prophylaxis [1].

For developing any vaccine, three things have to be taken care of: cost effectiveness, no or negligible side effect both on the host and the environment, and production of optimum immune response for longer period of time in the host to protect against diseases. Historically, the relative emphasis placed on each of these factors has been varied. Throughout the mid-20th century, when there was a great need and even with poor understanding of the immune system, the primary target of vaccine development was on efficacy, and a good number of formulations were developed using live-attenuated strains, heterotypic agents and killed pathogens [2]. With live attenuated vaccine, reversal of pathogenicity is always a big challenge. In this context, recombinant DNA techniques have enormous potential for the development of economical, safe and efficacious vaccines for the aquaculture industry. Aside from attenuated pathogens, two major categories of recombinant vaccines have been described. The first of its type is called "vectored" vaccines, consisting of either viral or plasmid expression vectors carrying genes for protective antigens from a given pathogen. The second type is recombinant subunit antigens produced using heterologous protein expression systems [3]. The first commercially available vaccine in fisheries was against the enteric red mouth disease (ERM, Yersiniosis) and Vibriosis introduced in the USA in the late 1970s. Both vaccines were inactivated whole cell vaccines and administered by immersion method [4]. Among viral vaccines, infectious pancreatic necrosis (IPNV) was the first commercialised fish

viral vaccine. Till date all the vaccines licensed are of live vaccine or attenuated vaccine, however, no recombinant vaccine has been licenced. But extensive work has been done on different fish pathogens for recombinant vaccine development. Different recombinant subunit vaccines based on infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) membrane glycoproteins have been tried on experimental basis [5, 6]. However, like any other vaccine, the recombinant vaccines are not free from criticism although it has many potential advantages.

In the recent past, significant contributions to the understanding of fish genomics and immunology along with understanding of microbial pathogenesis have taken place that are likely to enhance the development of vaccines and drugs for aquaculture. With improved and powerful scientific tools, new variations in the types of vaccines available are playing an increasingly important role in fish health management. These efforts are focused on producing the ideal vaccine economically, which must induce long lasting protection starting at an early age, prevent carrier formation, and be effective against a large number of pathogenic serotypes. These attributes of the ideal vaccine are most likely to be met either by a recombinant subunit vaccine [7]. With the advent of molecular cloning techniques in the 1970s, large-scale production of recombinant subunit antigens became possible using heterologous protein expression systems such as *Escherichia coli* and yeast. The ability to produce large amounts of purified antigen in non-pathogenic, single-celled organisms had advantages with respect to cost and safety, and led quickly to the development of successful vaccines for humans and animals [8, 9]. Finally, in the 1990s, plasmid expression vectors (the so-called "DNA vaccines") encoding microbial antigens offered still another promising approach towards vaccine development [10]. Otherwise known as genetic immunization, this process has been found to be highly effective against a wide range of pathogens (including those of fish), is extremely low in cost, and quite safe.

Recombinant vaccine strategies

Several genes from different pathogens have been cloned, expressed and purified to be tested as vaccine candidates. There are a variety of expression systems for antigenic protein components, such as bacteria, yeast, mammalian cells and insect cells, in which the DNA encoding the antigenic determinant can be inserted and expressed. However, several factors need to be considered before selecting an appropriate system for antigen expression. The level of expression obtained using each specific expression vector and promoter, the selection marker of choice, the presence or absence of post-translational modification by the recombinant vector, among others, are important criteria that modulate the efficacy of production of recombinant antigens as vaccines. Bacterial expression

systems are commonly in use as it is easy to handle and show high level of expression. However, for antigens in which post-translational modifications (e.g., glycosylation) are needed, the use of mammalian or insect cells need to be considered.

Types of recombinant vaccines

There are mainly three types of recombinant vaccines described: a. recombinant subunit vaccine, b. recombinant attenuated vaccine, and c. recombinant vectored vaccine.
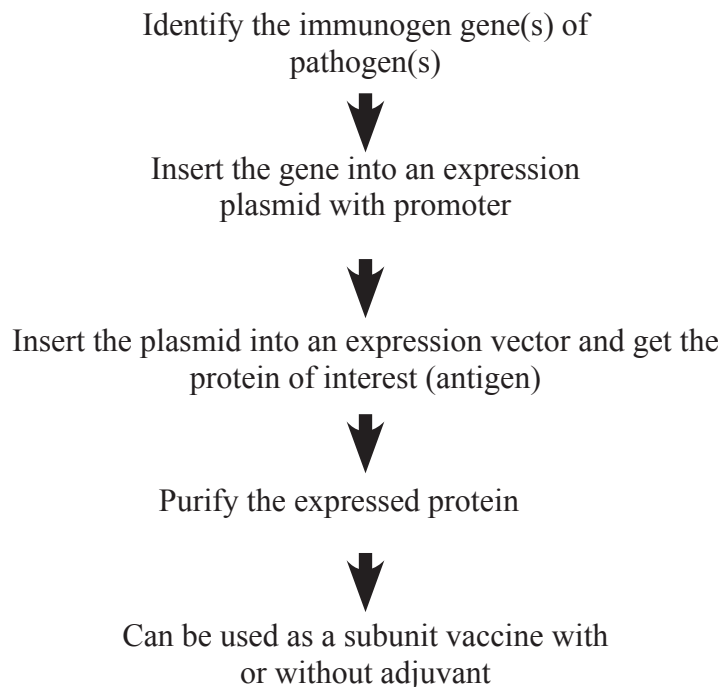
Recombinant subunit vaccine

Instead of the complete pathogen, subunit vaccines include only the antigens that best stimulate the immune system. Sometimes only an epitope (part of an antigen that can be recognized by antibodies or T cells) can be used. Because subunit vaccines contain only the essential antigens and not all the other molecules that make up the microbe, the chances of adverse reactions to the vaccine would be limited. Subunit vaccines can contain anywhere from 1 to 20 or more antigens. However, identifying antigenic candidates that best stimulate the immune system is a tricky, time-consuming process. One can make subunit vaccines in one of two ways: grow microbes in the laboratory and then use chemicals to break it apart and gather the important antigens, or manufacture the antigen molecules from the microbe using recombinant DNA technology. Vaccines produced this way are called "recombinant subunit vaccines."

The major drawback of this approach has been the difficulty of expressing recombinant viral and protozoan membrane antigens (which constitute the majority of vaccine candidates) in their native structural forms. Microbial systems (*E. coli* and yeast) can generate misfolded or incorrectly processed proteins that lack conformational epitopes required for the production of neutralizing/protective antibodies in the host [11, 12]. Similarly, the formation of protein aggregates (inclusion bodies) commonly seen following over-expression in bacterial systems is detrimental to native 3-dimensional structure [11]. Further, the complexity of expressing toxic proteins in bacterial expression system remains also as a challenge. On the other hand, the eukaryotic expression systems (insect and mammalian cells in culture) are more complex but accurate that renders proper protein folding and processing, although expensive. To date, this issue has hindered the development of recombinant subunit vaccines for some of the most important pathogens of farm-raised fish.

Initial work with subunit vaccines was not successful due to the rapid degradation of protein during processing, delivery, or in the animals. However, rapid advances were made to stabilize the antigens and many subunit vaccines were developed in the recent past. Highly successful examples of subunit vaccines are the IPNV VP2-based vaccine

from Microtek International and the ISAV recombinant hemagglutinin esterase gene from Centrovet [7]. Besides, recombinant rOmpR, an outer membrane protein of bacteria has been found to be an effective vaccine candidate against *Aeromonas hydrophila* infection in *L. rohita*. The vaccine formulation probably provokes humoral, cellular and innate immunity of the host. The protective response was more prominent when the antigen was administered with the modified adjuvant for a better and broad immune response [13]. Add to another example in the case of infectious pancreatic necrosis virus VP2 protein, regions encoding neutralizing B-cell epitopes have been mapped, and a protein expressed from VP2 cDNA in *E. coli* has been shown to render protection against IPNV in field trials with Atlantic salmon using a commercial multivalent vaccine (RPS = 60 %; Intervet Norbio) [14]. Some of the recombinant vaccines which are licensed in different countries are IHNV from recombinant G protein, licensed in Canada; spring viraemia of carp virus (SVCV) from recombinant G protein in baculovirus expression system, licensed in Belgium; infectious salmon anaemia virus (ISAV) from recombinant hemagglutinin esterase protein, licensed in Chile; IPNV from VP2 and VP3 capsid proteins and VP2 protein (Trivalent SRS/ IPNV/Vibrio) licensed in Canada and Chile, respectively [7].

Flowchart of subunit vaccine production:

Identify the immunogen gene(s) of
pathogen(s)

↓

Insert the gene into an expression
plasmid with promoter

↓

Insert the plasmid into an expression vector and get the
protein of interest (antigen)

↓

Purify the expressed protein

↓

Can be used as a subunit vaccine with
or without adjuvant

The table below details the progress made in brief at the experimental conditions with regard to subunit vaccines in aquaculture systems.

Table 1: Recombinant vaccines developed for fishes

Disease	Pathogen	Gene product	Fish	Reference
GCRV-GD108	Reovirus	VP4 (capsid protein)	Grass carp	[15]
AHNV	Nodavirus	Capsid	Atlantic halibut	[16]
IPNV	Birnavirus	VP2	Rainbow trout	[17]
		VP2/VP3 capsid protein	Rainbow trout	[18]
		VP2 based SVP	Rainbow trout	[19]
		Segment A polyprotein	Rainbow trout	[20]
ISAV	Isavirus	Hemagglutinin esterase (HE)	Atlantic salmon (*Salmo salar*)	[21]
SAV	Alphavirus	SAV-replicon	Fish cell lines	[22]
VHSV	Rhabdovirus	G-Protein	Fish cell culture (EPC and CHSE-214)	[23]
Furunculosis	*Aeromonas salmonicida*	A-layer protein	*Carassius auratus*	[24]
Edwardsiellosis	*Edwardsiella tarda*	GAPDH	Japanese flounder	[25]
Bacterial disease	*Aeromonas hydrophila* and *Aeromonas sobria*	Omp-G	European eel (*Anguilla anguilla*)	[26]
Motile aeromonas septicemia	*Aeromonas hydrophila*	OmpF	*Labeo rohita*	[27]

Disease	Pathogen	Gene product	Fish	Reference
Vibrio infection	*Vibrio sp.*	OmpK	*Epinephelus coioides*	[28]
Systemic disease in salmons	*Piscirickettsia salmonis*	Hsp60, Hsp70 and flagellar protein FlgG	CHSE-214	[29]
Marine bacterial infection	*Vibrio harveyi*	VhhP2	*Paralichthys olivaceus*	[30]
Motile aeromonas septicemia	*Aeromonas hydrophila*	OmpW	*Labeo rohita*	[31]
Motile aeromonas septicemia	*Aeromonas hydrophila*	ompTS	*Labeo rohita*	[32]
Motile aeromonas septicemia	*Aeromonas hydrophila*	Aha1 and OmpW	Common carp	[33]
Bacterial infection	*Aeromonas hydrophila* and *Edwardsiella tarda*	Omp48 of A. hydrophila	*Labeo rohita*	[34]
Edwardsiellosis	*Edwardsiella tarda*	OmpA	Common carp	[35]
Edwardsiellosis	*Edwardsiella tarda*	YaeT (omp85)	*Labeo rohita*	[36]

GCRV- Grass Carp Reovirus, AHNV- Atlantic Halibut Nodavirus, SAV- Salmon Alpha Virus, VHSV- Viral Hemorrhagic Septicemia Virus

Recombinant attenuated vaccine

Live, attenuated vaccines contain a version of the living microbe that has been weakened in the laboratory to make it avirulent. As a live, attenuated vaccine simulates a natural infection, these vaccines are good "inducer" of the immune system by eliciting strong cellular and antibody responses and conferring lifelong immunity with only one or two doses. Most common methods used for the attenuated vaccine production is to retain the pathogenic organism in adverse condition for long time so that it losses its original pathogenicity and can be used as a vaccine candidate. However, there is huge risk associated with this process of pathogenicity reversion. In this context recombinant technology can play a very good role of identifying all or key pathogenicity responsible genes of virus, bacteria, fungal or parasites and deleting it. By deleting the antigenic gene

(by making a mutant) the organism can be used as a good live attenuated vaccine. Below mentioned are some of the examples of deletion mutants experimented in various fish species as successful vaccine candidates in experimental conditions (Table 2).

Table 2. Mutant microbes experimented as vaccine candidates

Disease	Pathogen	Deleted gene	Fish	RPS	References
Yersiniosis	*Yersinia ruckeri*	lpxD (in-frame shift deletion)	Rainbow trout	≥ 84%	[37]
Vibriosis	*Listonella anguillarum*	sdhB (in-frame shift deletion)	Rainbow trout	Injection-84.2%, immersion-78.9%, and oral -76.3%	[38]
Edwardsiellosis	*Edwardsiella tarda*	esrB (error-prone PCR)	Turbot	Injection-80% and immersion-51.1 %	[39]
		Ugd (in-frame deletion)		76.7%	[40]
Streptococcosis	*Streptococcus iniae*	srtA (allelic exchange mutagenesis)	Nile tilapia	95.5%	[41]
Enteric septicaemia	*Edwardsiella ictaluri*	gcvP+sdhC (double gene deletion)	Channel catfish	100%	[42]
		Crp (in-frame deletion)	Zebrafish and catfish	100%	[43]
Vibriosis	*Vibrio alginolyticus*	vscO (in-frame deletion)	fish	74%	[44]
Francisellosis	*Francisella asiatica*	iglC	Tilapia	90%	[45]

Recombinant vectored vaccine

Recombinant vector vaccines are experimental vaccines similar to DNA vaccines, but the formulations use an attenuated virus or bacterium to introduce microbial DNA to the cells of the body. "Vector" refers to the virus or bacterium which is used as the carrier. In the process, the vector (either virus or attenuated bacteria) carries the gene of interest to insert the same to the cells (in case of virus) or display the antigens on bacterial surface. Recombinant vector vaccines closely mimic a natural infection and therefore, plays a significant role in stimulating immune response.

Vector vaccines have attracted very limited study in finfish aquaculture. A replicon vaccine based on the structural proteins of salmon pancreas disease virus has shown immunity against ISAV in salmons [21]. However, the limitation in this type of vaccine is the existence of pre-formed antibodies against the vector that reduces the production of antibodies against the foreign antigen. Table 3 summarizes a few pertinent experimental studies undertaken on vector vaccine in fish.

Table 3: Recombinant vector vaccines in fishes

Disease	Pathogen	Expression vector	Fish	Degree of protection	Reference
Edwardsiellosis	*Edwardsiella tarda*	*E. coli*	Zebrafish	63% RPS	[46]
Viral haemorrhagic septicaemia	VHSV	Auxotrophic *E. tarda*	Olive flounder	50% survival rate	[47]
Infectious pancreatic necrosis	IPNV	*Lactobacillus casei*	Rainbow trout	46% reduction in virus load	[18]
A. hydrophila and *V. anguillarum* infection	*A. hydrophila* and *V. anguillarum*	*V. anguillarum*	Turbot	-	[48]

Conclusion

Vaccination seems to be one of the best management measures to render long term protection against diseases. In spite of the fact that vaccines can be vastly useful in fish disease management, very few effective vaccines have been developed and commercialised world-wide. Most of the vaccines developed are mainly for the high valued fishes, i.e., salmons, trouts, etc. Hence, there is still opportunity in the sector

for the researchers to develop an effective and low cost vaccine. Most of the vaccines developed/commercialised are of killed or inactivated nature with a problem of efficacy or pathogenicity reversion. Recombinant vaccine technology has a potential role to play in the immediate future. Although only a few of the experimental trials have been successfully conducted on subunit or recombinant vaccines, it is high time to bring those to the level of commercialization for wider efficient applications.

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DNA vaccines for aquatic animals

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Introduction

The intensification of aqua farming led to diseases as a major problem globally. Pathogens constitute the most significant cause of destruction of nearly 10% of cultured aquatic animals, amounting to economic loss of 8-10 billion USD annually [1]. Disease prevention by vaccination is considered as the most appropriate means for controlling pathogens. DNA vaccines are third generation vaccines made up of a genetically engineered circular plasmid DNA (pDNA), to produce an immunological response by injection.The definition of DNA vaccination as provided by the Norwegian Biotechnology Advisory Board (2003) is "The intentional transfer of genetic material (DNA or RNA) to somatic cells for the purpose of influencing the immune system. The DNA vaccine has been a promising candidate in the defence particularly against intracellular pathogenic virus and bacteria that might require cell mediated antigen presentation to confer immunity. DNA vaccines have been developed not only for prophylactic purposes but also to target non-infectious health issues such as cancer and rheumatism [2,3]. Indeed, the emergence of the first commercialized DNA vaccine for fish had prompted further studies for the development of more efficacious vaccines to protect cultured fish against infectious diseases. This article describes in brief the general aspects, studies on fish pDNA vaccines, and concerns of safety and regulations of DNA vaccines.

Principle of DNA Vaccines

DNA vaccines consist of a DNA molecule, which is generally an expression plasmid, containing a specific gene that codes for only a selected protein which is expected to elicit an immune response. The concept of DNA vaccines has been experimentally demonstrated about two decades ago. In 1990, Jon Wolff of the University of Wisconsin found long-term expression of DNA plasmids injected in mice. In 1993, Dr. Margaret Liu from Merck Research Laboratories found that intramuscular injection of DNA from influenza virus in mice produced complete immune response [4]. Rainbow trout (*Onchorhyncus mykiss*) immunized against IHNV by DNA vaccination is the first report in fish [5].In 2005 a vaccine against IHNV infection in salmonids (Apex-IHN®, Novartis Animal Health) was the first DNA vaccine ever to be cleared for marketing by the Canadian Food Inspection Agency.

DNA vaccination strategy is based on the following principles:

- ✓ A gene encoding for specific antigen can be expressed in transfected cells

- ✓ An extraneous antigenic protein produced within the host cells can induce humoral and cellular immune responses.

Engineering a DNA vaccine

The basic steps include selection of gene of interest which codes for the proteins to stimulate immunity against the pathogen, called the transgene, isolation of selected transgene from the pathogen, designing and construction of plasmid vector with the gene of interest, then the plasmid is grown in bacteria, purified and delivered in to the host. Plasmid vector is the key part in the DNA vaccine design as it functions as a vehicle for gene delivery. The transcriptional unit in the plasmid consists of three portions, namely, the transgene sequences that express a protein antigen/s capable of inducing immune response, flanked by a eukaryotic promoter/enhancer and a transcription/polyadenylation sequence to promote gene expression in the vaccinated animals [6]. Viral promoters like Cytomegalo virus (CMV), Rous sarcoma virus (RSV) and Simian virus 40 (SV40) are used to promote the transgene kept under its control. DNA vaccine (pIRF1A-G) containing the promoter regions upstream of the rainbow trout interferon regulatory factor 1A gene (IRF1A) elicited protective immune responses in rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus (IHNV) [7]. The polyadenylation sequence is an essential aspect of gene expression, playing an important role in mRNA stability and translation and most vectors use SV40 poly A tail. The general properties of DNA vaccines are:

1. The immuno-protective antigen(s) should retain expression of the antigen at the site of injection and/or in an immunocompetent tissue.

2. The correct processing of immuno antigens so that the immunization elicits a native immune response similar to that of immunogens of pathogens.

3. Minimal required dose with ease of administration for immunization.

4. Should possess appropriate promoter/enhancer, duration of expression and persistence of the vaccine DNA in the injected animal.

5. It should have minimal environmental risk and no residual hitches in the animals at the time of marketing.

6. Should be relatively simple, quick to produce, stable at room temperature and easy to administer.

7. Capable of providing a broad, effective and protective spectrum.

8. Long lasting immune response and efficacy, duration of the immunity of the vaccine verified in live challenge experiments and in field trials.

9. Should provide maximum protection at different life stages of the host.

10. Should be cost effective to manufacture and to be easily licensed or registered.

11. No integration of the vaccine DNA into the host genome.

12. Vaccine should be safe for the fish and the end-user.

Molecular and cellular mechanism of action of DNA Vaccines

Even though the mechanism of development of the immune response in fish after DNA vaccination is poorly understood, DNA vaccines have shown to induce potent T and B cell immune responses against a variety of antigens [8]. DNA vaccines consisting of naked plasmid DNA, when delivered intramuscularly, result in gene expression of immunogenic proteins in the muscle tissue of the vaccinated fish. The system of a DNA vaccine requires the cellular machinery to replicate and trigger immune responses. After administration of the plasmid, it is usually taken up by a cell (myocytes), the DNA is transcribed and mRNA is translated to protein(s) by the cell's own apparatus. Expression of the gene encoding the antigen induces an immune response resulting in disease protection [9]. Briefly, at the immunization site, both the somatic cells (e.g. myocytes) and the proximate antigen presenting cells (APCs) get transfected and the production of the vaccine protein depend on transcription and translation of the administered DNA vaccine within the transfected cells. Thus, the foreign antigenic proteins are endogenously produced, intra-cellularly processed and their fragments (antigenic peptides) are exposed on the cell surface by the MHC class I and class II molecules involved in induction of cellular and humoral immunity. The DNA vaccines have been shown to elicit antibody and cytotoxic T lymphocyte (CTL) response (Fig. 1). Three mechanisms of immunogenicity of DNA vaccines are possible: (i) presentation of antigens by myocytes to CD8+ cells directly through their MHC class I pathway, (ii) direct transfection of APCs and (iii)

phagocytosis of transfected somatic cells by APCs which present the antigen to T cells. The DNA vaccine will be present and active for a short period of time and during this period, a large quantity of protein will be formed, giving rise to an immune response [10, 11, 12, 13].
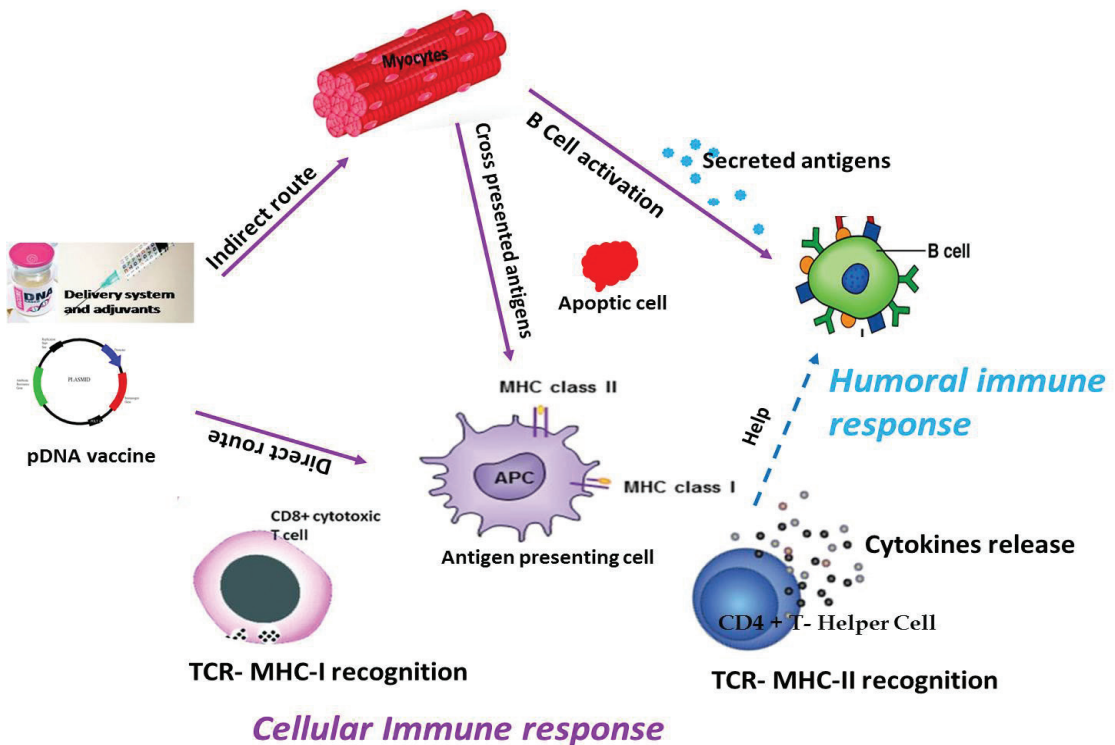
Fig.1. Schematic representation of Mechanism of Action of DNA vaccines

Delivery means for DNA vaccination

DNA vaccines may be administered by different routes: intramuscular (IM), intradermal (ID), oral or particle mediated administration. DNA vaccination through intramuscular delivery in fish is one of the promising vaccine applications to control fish diseases. Other means of delivery in fish are immersion, spraying, genegun and electroporation. Administering a DNA vaccine via the oral or immersion route is ideal for small and large numbers of fish as these routes reduce the labour required, minimize the stress to the fish and generate a significant mucosal immune response [14].

Adjuvants to enhance immunogenicity of DNA Vaccines

To increase the immunogenicity of DNA vaccine, aluminium salts, polysaccharides (zymosan, glucans, chitosan), liposomes, synthetic polymers , TLR agonists and plasmid encoded cytokines are used as adjuvants. IL-2, IFN-γ, IL-12, GM-CSF and IL-15 have been shown to modulate immune responses when co-encoded by the DNA vaccine [15]. Chemokines, transcription factors and/or co-stimulatory factors are assembled into the plasmid vectors for immune modulating effects. In Japanese flounder, interferon regulatory factor-1's (IRF-1) modulatory effect on early immune response has been reported [16]. To increase DNA vaccine potency and efficacy, one of the strategies is inclusion of molecular adjuvants, in combination with targeting carrier systems such as nano- and microparticles. Microencapsulation is a good choice for plasmid delivery. High protection with a relative per cent survival (RPS) of 83% associated with the production of neutralising antibodies that lasted for at least 60 days was observed in trout with the pcDNA-VP2 plasmid encapsulated with alginates after oral vaccination [17]. Other compounds tested as adjuvants and gene vaccine delivery systems are alginate, chitosan, liposomes, polycaprolactone and calcium phosphate in different fish species [18- 24]. There have been experiments with suicidal DNA vaccines for fish, where the plasmid vector includes a protein to induce apoptosis after an immune response has been triggered [25].

Factors influencing DNA vaccination

Due to large size and negative charge, nucleic acids have poor intrinsic transfection efficiency. The processes of gene transcription may be influenced by a variety of factors such as pDNA vector design, choice of promoter, pDNA concentrations, administration dose, fish age and size, water temperatures and route of administration. Transgene expression has been found to be higher in young, growing fish as it appears to favour the distribution of pDNA and transgene expression throughout the tissue in small size fish [26]. It has been well documented that the DNA topoform has a strong influence on the efficiency of transfection. While supercoiled DNA is reported as potent topoform, followed by open circular forms, linearized pDNA has shown to annihilate the expression of transgene [27]. The stimulation of PRRs may also induce responses that can be detrimental to transgene expression. The hallmark cytokines of the inflammatory response, TNF-α and IL-1β, have been shown to inhibit transgene expression *in-vitro* and *in-vivo* [28]. There are indications that excessive DNA concentrations may actually reduce transgene expression [29].

Status of DNA vaccines in aquaculture

DNA vaccination of fish has been shown to be efficient especially against novirhabdo viruses like viral hemorrhagic septicaemia virus (VHSV) and infectious hematopoietic virus (IHNV) in salmonids wherein the viral surface protein (glycoprotein, or G protein) acts as the protective antigen. As DNA vaccines provide a high level of protection against the rhabdo viruses, DNA vaccine techniques have been investigated in cultured fish and there is growing interest in the development of DNA vaccines for fish (Table 1). However, DNA vaccines against ISAV and infectious pancreatic necrosis virus have shown inferior protective effects compared to rhado viral DNA vaccines [30]. The first demonstration of IM injection of a plasmid DNA encoding the IHNV G gene into fish resulted in the transient expression of the encoded gene and generation of protective immunity against IHNV challenge [31]. Myocytes and mononuclear cells take up pDNA after administration [32] but despite a rapid initiation of uptake, the subsequent uptake is slow and cells along the muscle fibers have been shown to be transfected over a period of hours following injection. With very small fish this initial dispersion of a vaccine might be enough to ensure the perfusion of intact pDNA to more distant tissues, while in large fish the injected volume will mainly rest along the needle trajectory [32]. The transportation of pDNA from blood to other tissues has been reported for various fish species [32]. Plasmids have been recovered from sites such as liver, spleen, head-kidney, heart and intestine for some time after injection, but mainly persist at the site of injection. Degradation of the pDNA starts within five minutes following injection of mice, with as much as 95-99% of the initial pDNA amount degraded within 90 minutes [32]. The rate of degradation in the tissue of cold-water fish remains to be determined. The extent of histopathological changes at the injection site following IM DNA delivery in fish appears to increase with an increase in vaccination dose [33], but vaccination will generally induce only moderate local tissue damage in the form of degeneration of myocytes, haemorrhages and a transient influx of inflammatory cells [34] .

Table 1: Status of DNA vaccines against different fish pathogens

Pathogen	Host	Antigen	Delivery method	Protec-tion	Refer-ences
Viral Pathogens					
AHNV	Turbot	G of VHSV	IM	Yes	[35]
	Turbot	CP of AHNV	IM	No	[36]
CCHV	Channel catfish	ORF59, ORF6	IM	Yes	[37]
HIRRV	Japanese flounder	G	IM	Yes	[38]

Pathogen	Host	Antigen	Delivery method	Protec-tion	Refer-ences
IHNV	Rainbow trout	G	IM	Yes	[31]
	Salmon	G	IM	Yes	[39]
	Trout	G	IM	Yes	[40]
	Trout	G	IM	Yes	[41]
	Rainbow trout	G , suicidal gene	IM	Yes	[25]
	Rainbow trout	G-different geno-groups	IM	Yes – cross protec-tion	[42]
	Rainbow trout	G and PLGA	Oral	No	[43]
	Rainbow trout	G and PLGA	IM	Yes	[43]
	Rainbow trout	G and alginate	Oral	Yes	[44]
	Rainbow trout	VP2	Oral	Yes	[45]
IPNV	Salmon	A + VP2	IM	Yes	[46]
	Salmon	VP2	IM	Yes	[46]
	Trout	VP2	Oral	Yes	[17]
	Brown trout	VP2	IM	Yes	[47]
	Rainbow trout	VP2 and Alginates	Bath	yes	[17]
	Brown trout	VP2 and Alginates	Bath	yes	[17]
	Rainbow trout	VP2 and alginate	Oral	Yes	[17]
	Atlantic salmon	VP2; Segment A of TA strain	IM	No	[48]
	Rainbow trout	VP2 and Alginates	Bath and oral	Yes	[45,49]
ISAV	Atlantic salmon	HE	IP	Partial	[50]
	Salmon	HE	IM	Yes	[50]
LCDV	Japanese flounder	MCP with PLGA	Oral	Yes	[51]
Megalocyti-virus	Turbot	86-residue VP	IM	Yes	[52]
RSIV	Red seabream	MCP and an ORF569	IM	Yes	[53]

Pathogen	Host	Antigen	Delivery method	Protec-tion	Refer-ences
SAV	Atlantic salmon	E1 and E2	IM	No	[54]
SVCV	Common carp	G	IM	Yes	[55]
	Koi carp	G	IM	Yes	[56]
VHSV	Rainbow trout	G	IM	Yes	[26]
	Rainbow trout	G	IM	Yes	[57]
	Rainbow trout	G	IM or IP	Yes	[58]
	Japanese flounder	G	IM	Yes	[59]
	Trout	G	IM	Yes	[60]
	Trout	G	IM	Yes	[61]
	Trout	G	IM	Yes	[41]
	Olive/Japanese flounder	E. tarda as delivery vehicle	IM	Yes	[62]
VHSV and IHNV	Rainbow trout	G of VHSV and IHNV	IM	Yes	[41,63]
VNNV	Turbot	C	IM	Yes	[36]
Bacterial Pathogens					
E. tarda	Japanese flounder	Eta6-FliC chimeric protein	IM	Yes	[64]
	Japanese flounder	D15-like surface antigen	IM	Yes	[65]
	Japanese flounder	Eta2	IM	Yes	[66]
S. iniae	Japanese flounder	sagF, sagG ,sagI	IM	Yes	[67]
	Japanese flounder	Sia10 delivered by E. tarda	Oral/ Alginate imm.	Yes	[68]
F. psychroph-ilum	Rainbow trout	Hsp60, hsp70	IM	No	[69]

Pathogen	Host	Antigen	Delivery method	Protection	References
S. iniae and V. anguilla-rum	Turbot	Sia10 and OmpU	IM	Yes, cross-protection	[67]
V. alginolyti-cus	Red snapper	FlaA	IM	Yes	[70]
V. anguilla-rum	Asian sea bass	Porin gene, OMP38, chitosan	IM	Yes	[21]
V. harveyi	Yellow grouper	FlaA	IM	Yes	[71]
	Japanese flounder	DegQ and Vhp1	IM	Yes	[72]
V. parahae-molyticus	Black seabream	OMPK and chito-san	IM	Yes	[73]
Parasites					
Cryptocaryon irritans	Orange spotted grouper	iAg	IM	Yes	[74]
Cryptobia salmocitica	Atlantic salmon and rainbow trout	Metalloprotease	IM	Partly	[75]
Ichthyophth-rius multifiliis	Rainbow trout	Immunobiliza-tion antigens and cystein protease	IM, gene gun and air pres-sure	No	[76]

Virus : Atlantic halibut nodavirus (AHNV); Channel catfish herpesvirus (CCHV) ;Red seabream iridovirus (RSIV) ;Viral nervous necrosis viruses (VNNV);Infectious pancreatic necrosis viruses (IPNV); Viral haemorrhagic septicemia viruses (VHSV) and infectious haematopoietic necrosis viruses (IHNV); Spring viremia of carp viruses (SVCV); Salmonid alphaviruses (SAV);Infectious salmon anaemia viruses (ISAV); Lymphocystis disease virus (LCDV); Hiramerhabdovirus (HIRRV)

Bacteria: *E.tarda - Edwardsiella tarda ; S.iniae - Streptococcus iniae ; F.psychrophilum-Flavobacteriumpsychrophilum ; V.anguillarum- Vibrio anguillarum; V.harveyi - Vibrio harveyi ; V. alginolyticus - Vibrio alginolyticus ; V. parahaemolyticus- Vibrio parahaemolyticus*

Antigen : C - capsid protein; VP2- viral protein 2; G- glycoprotein; E2 - fusion protein; HE-hemaglutinin-esterase; MCP- Major capsid protein ; ORF-open reading frame

Delivery method: IM - intramuscular injection; IP- intraperitoneal injection Imm., immersion in water

Virtues of DNA vaccines

The merits and demerits of DNA vaccines are presented in table 2

Table 2: Advantages and disadvantages of DNA vaccines [8, 77 - 81]

Advantages	*Disadvantages*
➢ DNA vaccines capable of providing stronger immune responses than conventional vaccines. Due to long-term persistence of the antigenic protein, multiple booster doses are not needed.	➢ Difficulty for mass vaccination of small fish.
➢ DNA vaccine may conserve the structure and hence also antigenicity of a transgenic antigen/protein.They do not require complete knowledge of the pathogenic organism.	➢ Not efficient against pathogens possessing non-protein immunogens.
➢ There is no risk for infection or reversion of the virulent mutates of vaccination strain.	➢ Official distinction between DNA vaccinated animals and genetically modified organism (GMO) is not clear and long-term safety issues remain to be analysed.Regulatory standards yet to be available for DNA vaccines for farming animals. Hitches for DNA vaccines commercialisation.
➢ DNA vaccines are safer, more stable, and easy to handle. Ease of development, production /quality assurance and relatively inexpensive. DNA vaccines are easier to transport as there are no specific storage requirements.	➢ Potential side effects resulting in myositis, chromosomal integration, chronic inflammation at injection site and tissue destruction
➢ Antigen presentation by both MHC class I and class II molecules, thereby activation of both humoral and cellular immune responses.	➢ Risk of potentially disrupting normal cellular processes that could affect a cell's normal protein expression pathways. Risk of affecting genes controlling cell growth.
➢ Possibility of incorporating molecular adjuvants such as CpG motifs.	➢ Atypical processing of bacterial and parasite proteins.

Advantages	*Disadvantages*
➢ Multiple genes can be encoded in order to give protection against different variants to form broad spectrum multivalent vaccine. Ability to prepare vaccines for new pathogen variants rapidly and confer effective protection across serotype variants. ➢ Protective immunity over a wide range of temperatures.	➢ Testing results are affirmative in small animals, but less notable in larger animals as DNA uptake to cells apparently decreases with increases in body size. ➢ May induce immunologic tolerance by antigens expressed inside host body. ➢ DNA vaccines may have a relatively poor immunogenicity. Chances of an immune response against the DNA itself, or the DNA delivery vector exist and there would be no immune response due to non-expression of protein immunogens.

Challenges of DNA vaccines

i. Safety aspects

The safety of DNA vaccines is a source of apprehension in aquaculture. Safety issues are related to incorporation into the host genome. Pathological processes at the site of injection, distribution and retention of foreign DNA for extended periods in the internal organs and tumourigenicity are of concern to the public and the regulatory bodies. Although studies suggested that the injected plasmid DNA does not integrate into the genome of the host cells [31,82] as vaccine constructs, encoding pathogen antigens most likely persist for a shorter period due to the elimination of transfected cells by the fish immune system [26,43], Theoretically, such integration is possible, but perhaps seen very rarely. Prominent local reactions at the site of injection, prolonged antigen expression, muscle cell destruction and granuloma formations by 3 to 12 weeks post vaccination [83, 84], distribution of luciferase encoding gene to internal organs shortly after administration and extended period of its expression, up to 24 months, were noted [85, 86] and immune mediated destruction of antigen-expressing myocytes in rainbow trout following DNA vaccination [34] has been reported.

ii. Regulatory aspects

Safety aspects include potential effects on the vaccinated animals, the environment and the consumer [30, 87, 88]. Scientific data on these lines from the field are limited. Feeding experiments on the effect of DNA-vaccinated fish on mammals, the spread of a DNA vaccine in the environment by predatory animals, testing of the intestinal flora and analysis of the microbial flora in the environment of the vaccinated fish need to be conducted. The issue of plasmid persistence and chromosomal integration of DNA vaccine are of relevance for both safety and policy [88, 89]. Safety and regulatory uncertainties are related to distribution and degradation of the DNA after injection and there is a need for research on (i) the stability of the DNA vaccine, (ii) plasmid persistence, (iii) unintended immunological impacts and (iv) potential for integration of the pDNA into the chromosome of the recipient organism [89]. These uncertainties are of relevance for consumer acceptance and safety aspects and need to be taken into contemplation by appropriate authorities. To quote, as reference, The Canadian Food Inspection Agency (CFIA) through the authority of Veterinary Biologics Section (VBS) of the Animal Health and Production Division (AHPD) approved the IHNV DNA vaccine for commercial use in Canada in 2005 [90], after taking into consideration the following five aspects : (i) public perception and acceptance, (ii) regulatory and environmental concerns, (iii) risk-benefit, (iv) feasibility of producing the vaccine at a scale and cost appropriate for the fish industry and (v) intellectual property issues [91]. The European Medicines Agency (EMA) has drafted guidelines for the veterinary use of DNA vaccines [92] that include i) the possibility of pDNA integrating into the chromosome, ii) concerns about possible adverse effects on the immune-system, iii) risks posed by the additional use of genes encoding cytokines or co-stimulatory molecules or iv) undesirable biological activity by the expressed antigen itself. The guidelines prepared by the USFDA recommends that safety testing should include tests on vaccine immunogenicity, effects from cytokines and other immunomodulatory genes, auto-immunity, local reactogenicity, systemic toxicity, studies of bio-distribution, persistence and integration [93]. In Europe and Norway, due to the uncertainties with regard to the persistence of a DNA vaccine, the DNA vaccinated fish are labelled as a GMO [88, 94] and accordingly the producers also need to meet the requirements of the EU environmental legislation on the deliberate release of GMOs (Directive 2001/18/EC). In USA and Canada there are no requirements for labelling of food containing GMOs and they do not have specific GMO legislation.

Conclusions and future directions

Vaccines stimulate the immune system to fight against diseases and the application of these methods to control infectious diseases is of mounting importance. Hence sustainable development of aquaculture relies on disease prevention. Safe and

efficacious vaccines are critical for a viable aquaculture industry. Vaccines are required for sustainable development of the aquaculture industry worldwide. In Canada, an Infectious Hematopoietic Necrosis Virus (IHNV) DNA vaccine (Apex-IHN®), developed by Aqua Health Ltd. (Canada), was approved for marketing by the Canadian Food Inspection Agency on 15 July 2005. This approval of the first DNA vaccine for use in farmed fish against IHN in Atlantic salmon in Canada was a major leap in the field of DNA vaccines against fish diseases. However, while DNA vaccines used in fish have proven efficacious against the novirhabdo virus infections, not many effective DNA vaccines are available for a number of other infections. Hence the emphasis need to be given on studies to improve the efficacy of DNA vaccines, including improvement of the vectors and carriers to increase the uptake, enhanced presentation of transgene in antigen presenting cells, adjuvants to boost the response, delivery of and subsequent enhancement of immune responses. More efforts are needed to understand the mechanisms of distribution, transcription, translation and degradation of the pDNA after administration. The question of foreign gene integration from the plasmid into the genome of vaccinated fish remains to be resolved to ensure the safety of the end-user.

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RNAi mediated prophylactics for aquatic animals

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Introduction

RNAi (RNA interference) is an ancient naturally occurring cellular process where small RNAs regulate gene expression (developmental to adult stage) and provide innate immunity against invading viruses. Small RNAs are not only involved in transfer of genetic information from DNA to protein, but also act as regulators of other RNA transcripts and transposon silencing in the germ line genome in a sequence-specific manner. In this process, short RNAs interact with sequence specific target mRNA and degrade them through enzymatic way leading to inhibition of translational (protein synthesis) event. This phenomenon was discovered in the early 1990's in petunia plant [1]. After some years, a similar phenomenon was reported to have natural anti-viral activity in plants [2,3]. The pathway behind this mechanism was identified as RNAi in *Caenorhabditis elgans* by Fire et al. [4]. The regulation of genetic element occurs at different levels of genome function such as chromatin structure, chromosome segregation, transcription, RNA processing and translation. The outcomes of this regulation by small RNA's on gene expression are of inhibitory nature and so the whole mechanism is referred to as RNA silencing. Small RNAs act as specificity factors that bind directly to associated effector proteins to target mRNA molecules through base-pairing interactions.

Since the discovery of RNAi, it has contributed a lot to the understanding of gene functions across animal or plant kingdom; moreover, it also has great potential as a therapeutic and prophylactic molecule. RNAi has been accepted as a versatile tool for application in reverse genetics to high throughput screening of drug targets. When industrialization increases and the human race advances to more sophisticated world of easiness, there is a parallel increase in new viral pathogen or disease that needs to be addressed. Viruses are known for their ability to mutate more frequently than any other pathogen, and the biological factors required for the replication machinery are all from host the itself. This property of virus creates limitations for the use of therapeutic drugs. Furthermore, mutation rates are as high as 10^3 errors per nucleotide per genome replication [5] favors viruses sequence diversity which allows them to evade host immune responses. As a result, there is an increase in the incidence of resistance to available anti-viral drugs.

Mechanism of silencing

RNA mediated gene regulation or gene silencing involves different types of small RNA molecules. Some of the types and their functions are shown in table 1 [6]. Of these small RNAs, miRNA and siRNA are predominantly found across both plants and animals and are derived from double-stranded precursor molecules. miRNAs are of endogenous in origin as they are available at every stage of the organism's life but mainly during the developmental stages of both plants and animals. miRNA sequences are encoded in the genome itself and they are processed from the precursor into small miRNAs inside the nucleus and exported to the cytoplasm for further sequence specific target binding and cleavage of other RNAs/mRNA transcripts.

Types of RNA	Length (nt)	Function
Micro RNA (miRNA)	19 – 25	Translational repression
Small interfering RNA (siRNA)	19 – 21	Target mRNA cleavage
Trans-acting siRNA (tasiRNA)	21 – 22	mRNA cleavage
Small-scan RNA (scnRNA)	~28	DNA elimination
Repeat-associated siRNA (rasiRNA)	24 – 27	Transposon control transcriptional silencing
Piwi-interacting RNA (piRNA)	26 – 31	Transposon control in germ cells

Table 1: Different types of small RNAs (Source:[6])

Unlike miRNA, siRNAs are derived from the long linear double-stranded RNA which are either introduced into the cytoplasm or taken from the environment. More recently, other sources of endogenous siRNAs have been reported by Golden et al. [7]. These include convergent mRNA transcripts and other natural sense-anti sense pairs, duplexes involving pseudogene-derived antisense transcripts and the sense mRNAs from their cognate genes and hairpin RNAs [8]. Hence siRNAs not only derive from foreign nucleic acids but also arise from endogenous genomic loci with a nuclear phase of RNA processing, which is not seen in exogenous siRNAs. Once inside the cytoplasm, the long double-stranded precursor molecule undergoes processing by Dicer, a type of RNase III enzyme (dsRNA specific nucleases) into small ~21 – 23 nt duplex RNA with ~2 – 3 nt at 3'overhangs, which is known as siRNA. This is further loaded into an enzyme complex

called RISC (RNA induced silencing complex) containing Ago proteins (Argonaute), an effector molecule. siRNA duplex binds to this Ago protein through one of the strands termed as "guide strand" and the other strand is termed as the "passenger strand" which is degraded once inside the RISC. siRNA guide strand further directs RISC to bind target RNA/mRNA molecule through complementary base pairing and degrad. RNA degradation is induced by the PIWI domain of Ago protein, which acts as a "slicer" with very precise activity: the phosphodiester linkage between the target nucleotides that are base paired to siRNA residues 10 and 11 (counting from the 5' end) is cleaved to generate products with 5' monophosphate and 3' hydroxy termini [9]. After this initial cut, cellular exonuclease starts degrading the fragments. Fig.1. represents a summary of RNAi mechanism. Thus a gene transcript is completely degraded irreversibly resulting in the silencing of the gene product itself. Mismatches found at or near the center of siRNA/target duplex leads to suppression of endonucleolytic cleavage. In some cases, siRNA Ago proteins lack endonuclease activity even though RISC is perfectly paired with the targets. Whether targets are partially mismatched or si-RISC complex has inactive endonuclease can still be silenced at post transcriptional level involving translational repression or exonucleolytic cleavage in a similar way of miRNA silencing.

Fig. 1. RNAi mechanism - Overview
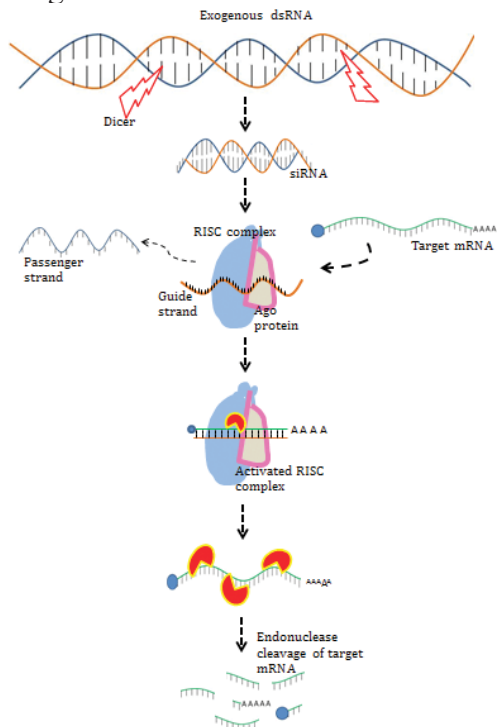
Designing of dsRNA/siRNA

As of today a number of insilco tools are available for effective designing of siRNA sequence from the target mRNA sequence. There are two ways of generating siRNA. One is to directly chemically synthesise siRNA as short oligo nucleotides and the other way is to clone siRNA sequence into a T7 RNA polymerase-dependent dual/ single promoter containing vector systems, which can be used to generate siRNA either in vivo or in vitro methods. Some of the siRNA design service providers are shown in table 2. But in the case of dsRNA synthesis, there is no online design program available yet primers are designed from mRNA sequence and the resulting amplicon sequence is analyzed using siRNA designing tool. All the parameters required to act as viable siRNA should be present in the newly designed amplicon sequence. If there's no siRNA-like sequence available then different primer pairs need to be tried out. This selected primer is PCR-amplified and the product is cloned on to a suitable vector system downstream of T7 promoter. Cloned plasmid is transformed into a suitable bacterial host such as HT115 (DE3) – mutant for RNase III enzyme. The recombinant clones can further be scaled up and in vivo dsRNA synthesis is induced with known concentrations of IPTG and the synthesized dsRNA is extracted, which will be further used for silencing the respective gene. This dsRNA once inside the target cells/organs will be processed into siRNA using host dicer enzyme and forms the RISC complex with the target RNA molecule leading to degradation. On the other side, *in vitro* synthesis of dsRNA is done by linearising the cloned plasmid and using that as template for T7 RNA polymerase, dsRNA is synthesized in a PCR reaction.

URL	Provider
http://rna.tbi.univie.ac.at/cgi-bin/RNAxs	University of Vienna
http://katahdin.cshl.org/html/scripts/main.pl?link=resources&content=resources	Cold Spring Harbor laboratories
http://dharmacon.gelifesciences.com/design-center/	GE Healthcare Dharmacon Inc.
https://rnaidesigner.thermofisher.com/rnaiexpress/	Thermo Scientific
http://sirna.wi.mit.edu/	Whitehead Institute for Biomedical Research

Table 2. Online siRNA design tools.

RNA interference in shrimps

RNA interference (RNAi) is found to be actively available in arthropods generating antiviral immunity [10. 11]. In contrast to the volume of data available for insect's RNAi,

only very little is known about crustaceans. In shrimp, RNAi mediated gene silencing was reported for the first time by Robalino and co-workers where, *in vivo* administration of dsRNA into *Litopenaeus vannamei* gave protection against viral infection. This denotes that marine invertebrates were able to induce antiviral immune response against viral infection [12, 13]. dsRNA induced gene silencing mechanism has been reported in several shrimp species, which denotes the very existence of RNAi machinery.

Dicer enzyme belongs to evolutionarily conserved family and found in many organisms such as animals, plants, fungi etc. Depending on the complexity of the organism there might exist one or more forms of dicer-like enzymes. For example, *Arabidopsis thaliana* has four dicer proteins, *Oryza sativa* five [14], and *Caenorhabditis elegans* one [15]. Similarly, in shrimp species like *Penaeus monodon* (PmDcr1) and *Marsupenaeus japonicus* (MjDcr1) only one form of dicer gene has been reported, whereas in *Litopenaeus vannamei* two types of dicer-like genes (LvDcr1 and LvDcr 2) have been reported [16-18]. Although several forms of shrimp dicers are being detected, it still remains unclear whether these proteins involve and possess functional activities in the processing of long double-stranded RNAs.

Dicer proteins have been found to act together with other dsRNA binding proteins in order to elucidate Argonaute – RISC complex such as HIV-1 transactivating response (TAR) RN-binding protein (TRBP) and a protein activator of PKR (PACT) in humans [19-21]. Much similar kind of TRBP was also reported in *Fenneropenaeus chinensis* (Fc-TRBP1-3) and *M. japonicus* (Mj-TRBP1-3) by Wang et al. [22, 23]. Phylogenetic analysis of TRBP sequence from all species showed that these sequences are highly conserved in terms of functionality with other members of the same family of dsRNA binding proteins. In higher organisms eukaryotic Initiation Factor 6 (eIF6) is found to be a component of RISC in RNA mediated silencing process [24]. Surprisingly, similar homologues of eIF6 were reported in *F. chinensis* (Fc-eIF6) and *M. japonicus* (Mj-eIF6). Further, knockdown of eIF6 and TRBP genes resulted in loss of dsRNA mediated gene silencing [22, 23].

Argonaute plays a key role in RISC and it was the first protein to be identified in penaeid shrimp RNAi pathway [25]. This protein family includes two sub-classes of proteins, namely the AGO and PIWI subfamilies. PIWI is mostly found to be associated with miRNA mediated gene silencing in germ line cells during developmental stages [26]. PIWI subfamily of argonaute proteins has not been reported in shrimps, but two isoforms (Pm-AGO and Pem-AGO) of AGO subfamily was reported in *P. monodon* whereas two different argonaute proteins (Lv-AGO 1 and Lv-AGO 2) were reported in *L. vannamei*. To understand the functional role of Dicer and Argonaute proteins in shrimp antiviral immunity, several studies have been performed to know the abundance of these two gene transcripts during viral challenges.

Whether it's a drug or viral pathogen entering the cell, there should be a host factor responsible for recruiting these molecules inside the cell's cytoplasm, called the receptor molecules. In higher vertebrates, the exogenous nucleic acids of viral origin or synthetically delivered nucleic acids are detected by pattern recognition receptors (PRRs) which belong to two super families, namely Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and the transmembrane Toll-like receptors (TLRs). In shrimps RLRs have not been reported yet, whereas TLRs have been reported in *L. vannamei*, *F. chinensis*, *P. monodon* and *M. japonicus*. However, available information on shrimp TLRs is not enough and also doesn't provide any clear picture of their role in detection of exogenous dsRNA; hence further studies are required to know the insights of dsRNA binding patterns and recognition by host RNAi pathway.

RNA interference – as a prophylaxis measure

RNAi mediated gene silencing can be used as a prophylaxis measure to reduce or inhibit several viral infections by using host RISC complex and dsRNA/siRNA to guide sequence specific cleavage of viral gene transcript and to some extent host genes transcript involved in intracellular trafficking of viruses. There are reports available in using RNAi to encounter viruses affecting shrimp farming. Sequence specific dsRNA induced RNAi has been used to inhibit viral replication in shrimp against the white spot syndrome virus (WSSV), the Taura syndrome virus (TSV), the yellow head virus (YHV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV). Robalino et al. [13] reported that dsRNA against TSV protease inhibited TSV replication in a sequence-specific way when compared to controls which showed complete mortality at 5 dpi. dsRNA specific to shrimp endogenous gene LvRab 7, also rendered protection against TSV infection in *L. vannamei;* in this case the host gene product acts as the receptor molecule in transporting the viral particles inside the cell [27]. Specific dsRNA against genes encoding structural and non-structural proteins of IHHNV showed reduction of virus replication at 8 dpi in *P. monodon* [28] (Attasart et al. 2010). In another study, Ho et al.[29] reported that treating dsRNA 12h before IHHNV challenge and additional treatments at 3 and 6 d post viral infection showed a high inhibition of IHHNV in challenged animals at 5, 8 and 10 d post challenge, in contrast control animals which showed high IHHNV levels by 5 d post challenge.

Of the sequence specific dsRNA targeted against genes encoding helicase, polymerase, protease and the structural proteins GP116 and GP64 of YHV, dsRNA against non-structural genes showed higher YHV inhibition [30] . In a similar study dsRNA against YHV protease showed 0% mortality of shrimp compared to 90% mortality in controls at 10 d post challenge [31] . In another study, dsRNA against YHV protease were

given at 3, 6, 12 and 24 h to *P. monodon* post viral challenge controlled YHV replication up to 12h post challenge [30]. Whereas unrelated dsRNA-GFP showed no inhibition of virus, denoting that dsRNA is sequence specific in nature. The next most lethal virus is WSSV inducing mortality in 3 – 4 days of infection. Treating shrimps with dsRNA specific to VP28, VP281 and protein kinase encoding genes showed effective blocking of WSSV replication and thereby confers protection. Similarly, oral administration of bacterially expressed dsRNA specific to VP28 of WSSV rendered protection to *P. monodon* challenged with WSSV.

dsRNA specific to VP28 or VP26 reduced susceptibility of *L. vannamei* to WSSV and survivors exhibited reduced susceptibility in subsequent re-infections. The efficacy of dsRNA targeting structural (VP28 and VP281) and non-structural genes (RR1 and DNA Pol) of WSSV was compared. The experimental results indicated that targeting of combined VP28 and RR1 genes effectively reduced the viral replication rate and thereby increased the survival of the host. Suppression of host-endogenous gene, namely PmRab 7, using dsRNA specific to that inhibits WSSV infection in *P. monodon*. Likewise suppression of host Glucose transporter 1 using sequence specific dsRNA reduced the mortality of *L. vannamei* challenged with WSSV. dsRNA against WSSV non-structural genes (orf89, WSV191) and structural genes (VP28, VP26) were analyzed in *L. vannamei* challenged with WSSV. The results suggest that orf89, VP28 and VP26 are highly effective in inhibiting virus replication and thereby increasing the survival ability of challenged shrimps. In a recent study, shrimps were challenged with WSSV and treated with recombinant baculovirus displaying VP28 and encoding dsRNA synthetic gene specific to RR2 (Bac-VP28-RR2) was compared to treatment with recombinant baculovirus displaying only VP28 (Bac-VP28). The result showed Bac-VP28-RR2 system showed 33% low cumulative mortality at 14 dpi than Bac-VP28 system. Sequence specific silencing of VP9 gene of WSSV, displayed a significant survival rate of 80% at 25 dpi when *Macrobrachium rosenbergii* challenged with WSSV. All these results collectively indicate that dsRNA can be used as a therapeutic agent with a proper delivery system.

Conclusion

Though RNAi is proved to be functional in shrimp, it acts more like a prophylaxis than therapy. It willbe more useful in hatchery for producing virus free larvae. However, the economy part of it needs to be looked into. Secondly, the culture practice at present is dominated by Pacific white shrimp for which development of SPF stock is possible and hence this prophylaxis is not necessary. For cultured shrimps, the delivery mechanism is a major challenge. Experiment conducted in laboratory conditions indicate maximum 40-45% survival upon challenge of dsRNA treated animals (unpublished results). However, the same has to be again verified in field condition to see the optimum survival numbers.

As has already been mentioned, the economic aspect of it has also to be considered in culture condition to finally prove the feasibility of its application. Therefore, a lot more research is required to prove this as an effective technology and protect against virus infection.

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Adjuvants for Development of Fish Vaccine

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The aquaculture industry produced globally 65 million tonnes in 2014, representing USD 150 billion. Aquaculture is a fast growing industry (10 to 12% per year). It is expected that aquaculture should produce over 100 million tonnes of fish in 2050. As per the recent information, fish from aquaculture represent 40% of the whole fish consumption globally. It is expected that in 2025, 50% of the fish consumption besides from aquaculture. More than 30 species of fish are farm produced, besides shellfish and crustaceans [1].

Until 1980, very few fish vaccines were used in aquaculture. In 1982, vaccines only existed for 2 fish diseases (Enteric redmouth disease (ERM) and *Vibrio anguillarum*). Today, vaccines have been developed for more than 25 diseases of fish. Mass vaccination started in the 1990s in the salmonid industry, especially in Norway. Before the generalization of the use of fish vaccines, antibiotics were used extensively to prevent bacterial diseases in fish production. The use of new vaccines for salmons allowed a strong reduction of antibiotic use and a fast development of the industry. It is considered that introduction of mass vaccination in the salmonid industry is one of the major success stories in the growth of the global salmon farming [2]. It allowed the salmonid production to grow from a few hundred thousand tonnes during the early 1990's to more than 1.3 million metric tonnes in 2012 (Figure 1).
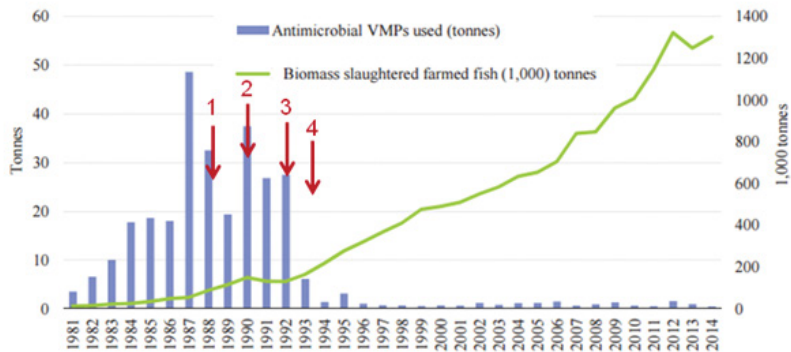
Figure 1 : *Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway* (Introduction of 1- Vibrosis vaccine, 2- Furuncolosis vaccine, 3- Oil-based vaccine, 4- Combination vaccine). NORM NORM-VET 2014

The role of the oil adjuvant in the success of vaccination of salmonids is important. It is indeed the stability and slow release of the adjuvanted antigen that allows single intra-peritoneal injection to protect through the 2 to 4 years grow-out period of salmons. This property made vaccination an economical option for preventing disease and led to almost universal adoption by salmon farmers within a few years [2].

Today, injectable vaccines for salmonids are usually adjuvanted with water in oil emulsion adjuvants. This practice of vaccination by intraperitoneal injection has been slowly transferring to non-salmonid species. This is an important transition as the major growth in finfish aquaculture is now occurring in warm-water species such as tilapia.

Adjuvants for injectable fish vaccines

Intraperitoneal (IP) injection (Figure 2) of 0.1ml to 0.2ml of water in oil vaccine is highly efficient and induces high and long term protection. Specific devices are available and injectable fish vaccines are extensively used in fish farming.

Figure 2: Intraperitoneal injection in trout.

Commonly used fish vaccines are based on inactivated bacterial or viral antigens (Table 1). New generation vaccines comprise attenuated or DNA antigens, but still represent a very small market share.

Pathogens	Vaccine name	Adjuvant
Multivalent bacterial and viral pathogens *(Aeromonas salmonicida, Vibrio salmonicida, Infectious Pancreatic Necrosis Virus, ...)*	Alpha Ject® range (PHARMAQ)	Mineral oil
Salmon pancreas disease virus	Norvax® Compact PD (Merck Animal Health)	Montanide™ ISA 763A VG
Aeromonas salmonicida	AQUAVAC® FNM (Merck Animal Health)	Montanide™ ISA 711

Table 1: Examples of injectable vaccines

Injectable inactivated vaccines are usually formulated with water-in-oil emulsion adjuvants to induce long term efficacy. A water-in-oil vaccine formulation is a dispersion droplets of the antigenic aqueous phase in a continuous phase of oil. As fish are sensitive to IP injection, oil adjuvants must be selected carefully to avoid viscosity and injectability issues (especially in cold water fish) and local reactions at the site of injection, such as melanisation and adhesions in the peritoneal cavity [3]. Such reactions should be avoided as they induce a loss of economic value of the fish. The type of oil (mineral, metabolizable, synthetic) and the quality of the oil are critical to ensure the safety and efficacy of fish vaccines. Metabolizable oils are usually safer than mineral oil for fish vaccines, but mineral oils can induce higher antibody titers and can be used to induce stronger cell mediated immunity.

Montanide™ adjuvants

Montanide™ range of water-in-oil adjuvants (Figure 3) has been used for fish vaccination worldwide. In particular, Montanide™ ISA 763A VG is a metabolizable oil-based water-in-oil adjuvant that has been shown to be safe and highly efficient for injection of diverse fish species, such as salmons, trouts, tilapia, seabass, turbot, catfish… [4-6]. This adjuvant has been used for commercial vaccines formulation in the last decades.
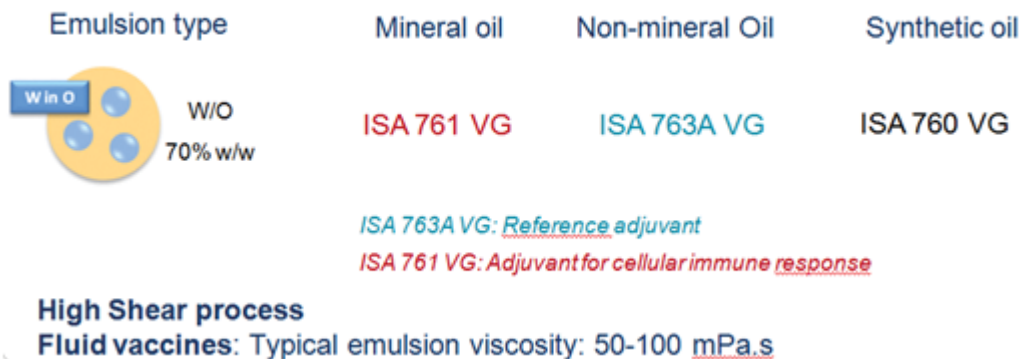
Figure 3: Montanide™ range for injectable fish vaccines

Montanide™ ISA 763A VG is a safe adjuvant that induces only minor reactions after injection. In a safety study for sutchi catfish *Edwardsiella ictaluri* vaccine, Montanide™ ISA 763A VG was formulated with inactivated antigen (10^9 CFU/dose) and 0.1ml of vaccine was injected to 2x30 catfish of 15 to 30g. The fish were slaughtered at D21 post injection and local reactions were assessed following Spielberg scoring scale (score 0 (no reaction to score 6 (global adhesion to the organs)). 75% of the fish had score 0 reaction, and no fish showed adhesion above score 1 (Figure 4).

Treatments (volume injected)	Type of local reaction (%)	
	Score 0 (no lesion)	Score 1
Antigen alone 10^9 CFU/ml	98 ± 2	2 ± 2
ISA 763 A VG with 10^9 CFU/ml	77 ± 8	23 ± 8
ISA 760 VG with 10^9 CFU/ml	77 ± 5	23 ± 5
Control (saline buffer)	98 ± 2	2 ± 2

Figure 4: No lesions above score 1 were observed in adjuvanted groups. Montanide ISA 763A VG is safe for fish vaccination.

In another study, the use of Montanide™ ISA 763A VG in a turbot vaccine against *Edwardsiella tarda* increased strongly the duration of immune response compared to non-adjuvanted vaccine [4]. At 1 month post injection, 100% of fish vaccinated with the adjuvanted vaccine were protected, compared to 80% in the non-adjuvanted group. At 6 months post-injection, 90% of fish vaccinated with the adjuvanted vaccine were still

protected, compared to only 20% in the non-adjuvanted group [4]. These results and others show that the use of adapted water in oil adjuvant is necessary to protect fish on the long term with only one injection.

New generation of injectable adjuvants, such as ligands or toll like receptors, are currently investigated in order to trigger specific immunological processes [7]. But as of today, oil adjuvants, when wisely chosen, still represent the most commonly used adjuvants; thanks to their satisfactory efficiency with a minimal risk ratio.

Adjuvants for immersion and oral administration of fish vaccines

Even though IP vaccination has demonstrated its efficiency, this route of administration is labour intensive, requires trained vaccination teams, and cannot be performed when very small or very large fish are concerned.

To avoid these technical issues, immersion and oral vaccination are being considered. Immersion consists in dipping the fish in a bath containing a vaccine for a few minutes. Oral administration consists in mixing the vaccine with the fish feed. Both methods are easier to implement than injection, but their efficacy has been until now limited. They are usually used as a complement to boost injectable vaccines [8], or for vaccination of juveniles when injection is not yet possible. Furthermore, field immersion and oral vaccines for fish do not usually contain adjuvants. That is why, the development of immersion and oral vaccines for fish requires dedicated adjuvants or formulations to improve their efficacy.

Adjuvants for immersion vaccines should be aqueous adjuvants that can be added to the immersion bath. Montanide™ IMS adjuvants are aqueous adjuvants composed of a micro-emulsion and containing an immunostimulating compound. It was shown that immersion vaccination against *Yersinia ruckeri* in rainbow trout was improved by the addition of the micro-emulsion adjuvant Montanide™ IMS 1312 VG [9]. This study showed that the vaccine against yersiniosis formulated with Montanide™ IMS 1312 VG induced a strong and long-term humoral and cellular immunity, and that the addition of adjuvant allowed reaching above 90% of protection against the disease after challenge, over 10 weeks after vaccination.

Developing efficient oral vaccination for fish would allow mass vaccination of the fish and a strong reduction of the workload necessary for fish vaccination. It would also limit considerably the risks of reactions after vaccination. However, as of today the efficacy of oral vaccination is not sufficient to replace vaccination by injection.

An option to enhance the efficacy of oral inactivated or subunit vaccines would be to improve the formulation of these vaccines. Oral vaccines must be mixed with feed to be administered to the fish. Vaccine can be lost in water and antigen may also be destroyed in the gastrointestinal tract of the fish. Formulations for oral vaccines should thus contain a gastro-protective matrix for the antigen, which should be able to stick to fish pellets in water until it has been swallowed by the fish and protect the antigen in the acidic part of the fish gastrointestinal tract. In addition, immunostimulants that activate the mucosal immune system could be added. Such formulations are being developed and tested to improve oral vaccines.

The development of new, efficient and safe fish vaccines is necessary to ensure an on-going growth of the aquaculture industry and a reduction of the use of antibiotics and anti-parasitic drugs used in fish farming.

The use of appropriate oil adjuvants allows the formulation of safe and protective one-shot injectable fish vaccines. New technologies to pinpoint specific immune responses are being investigated but are not used in the field yet. The development of more efficient immersion and oral vaccines, closely linked to the development of dedicated adjuvant technologies, should allow an efficient mass vaccination of fish in the coming years.

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Iv. Immunostimulants

Immunostimulants in Aquaculture

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Introduction

Aquaculture is the highly dynamic and fastest growing food producing sector. The contribution of aquaculture to fish production is steadily increasing. The increasing demand for fish products and commercialization of aquaculture leads to intensification of aquaculture. These conditions tend to adversely affect the health of the animals and produce poor physiological environment and subsequently increases the susceptibility to infectious disease. Diseases have become a major constraint by causing mass mortality and severe economic loss. To overcome the disease problem, generally antibiotics, chemotherapeutics and disinfectants are used in aquatic systems, which in turn lead to drug resistance, human carry over, bioaccumulation, and pollution to the aquatic environment. Vaccination is also a useful prophylactic treatment, but due to its limited availability and pathogen specific protective action, much attention has been diverted towards the application of immunostimulants.

Properties of Immunostimulants

- Good efficacy

- Wide spectrum of activity

- No toxic side effects

- No accumulation of toxic residues

- No environmental impact

- Mainly enhance nonspecific immune system

- Easy to apply for larvae of fish and shrimp

- Cost effective

Classification of immunostimulants

The nonspecific or innate immune response is the first line of defense against invading pathogens and fish and shellfish are dependent on innate immune response for protection. Immunostimulants are dietary additives that enhance the innate (non-specific) defense mechanisms and increase resistance to specific pathogens. Studies

on the immune-stimulatory effects of various compounds have found immune enhancing potential, by mounting nonspecific immune responses in fish and shellfish. Immunostimulants considerably improve the growth and survival rates and disease resistance in aquaculture systems and these effects depend on the structure and function of different immunostimulants. Different types of immunostimulants, such as synthetic chemicals, bacterial derivatives, animal and plant extract, nutritional factors, antimicrobial components and nucleic acids, evaluated in aquaculture are listed below (Table 1).

Table: 1   Immunostimulants assessed in Aquaculture.

Source	Type of immunostimulant
Synthetic Chemicals	• Levamisole • FK-565 • MDP (Muramyldipeptide).
Bacterial derivatives	• ß-glucan • Peptidoglucan • FCA • LPS (Lipopolysaccharides) • Clostridium butyricum • Chromobactersterohalis • *Vibrio anguillarum* cells
Polysaccharides	• Chitin • Chitosan • Lentinan • Schizophyllan • Oligosaccharide
Animal Extracts	• Ete(Tunicate) • Hde(Abalone) • EF203 • Firefly squid • Quillajasaponin (Scaptree) • Glycyrrhizin (licorice)

Nutritional Factors	• Vitamin C
	• Vitamin E
	• Carotenoids
Hormones	• Lactoferrin
	• Interferon
	• Growthhormone
	• Prolactin
Nucleic acids	• Nucleotides
	• CpG ODN

Mechanism of action of Immunostimulants

Immunostimulants are considered as alternative for antibiotics and chemotherapeutics. Immunostimulants, also known as immunostimulators (drugs and nutrients), stimulate the immune system by inducing or increasing the activity of any of its components. Immunostimulants are products of either natural or synthetic origin with different chemical characteristics and varied modes of action [1]. Immunostimulants are substances that activate the immune system of animals to make them more resistant to microbial infections [2]. Immunostimulants deliver disease resistance by enhancing non-specific immune system. They activate/stimulate/enhance the immune components such as phagocytic cells, natural killer cells, complement, lysozyme, activity of T cells and B cells, inflammatory agents, activity of Macrophage. In case of shrimps, it enhances the phagocyte activity, phenol oxidase activity, SOD activity, total haemocyte count, respiratory burst activity, etc.

Use of Immunostimulants in aquaculture

Synthetic chemicals

Levamisole

Levamisole, which is a synthetic phenylimidathiazole, has been approved by US Food and Drug Administration (FDA) for treatment of helminths in ruminants. In aquaculture, the immunostimulatory effect of Levamisole is considerable against bacterial infection and parasitic infestation. It improves the non-specific defence mechanism by enhancing the cell mediated cytotoxicity, lymphokine production, suppression of cell function and stimulation of phagocytic activity of macrophages and neutrophils. It has been shown to have the ability to upregulate the non-specific immune response of carps,

rainbow trout and gillhead sea bream [3].The immunostimulatory effects of Levamisole by oral and immersion methods also show increased disease resistance [4,5].

Immunoactive peptide (FK-565)

FK-565 (heptanoyl-y-d-glutamyl-(l)-meso-diaminopimelyl-(d)-alanine) is a lactoyltetrapeptide (FK-156) isolated from cultures of *Streptomyces olivaceogriseus.* It is shown that injection of FK-565 induces resistance against *Aeromons salmonicida* in rainbow trout, by activating the phagocytes [6, 7]. In vitro immunization also shows elevated humoral antibody titers when *Yersina ruckeri* or *A. salmonicida* O- antigen preparations were mixed with FK-565 [4].

Bacterial derivatives

Muramyl dipeptide (MDP)

Muramyl dipeptide (MDP), N-acetyl-muramyl-L-alanyl-D-isoglutamine, is a derivative from mycobacterium. It stimulates the immune system by enhancing the activity of macrophages, B lymphocytes and alternative pathway of complement [4]. Injection of MDP had shown increased resistance against *A. salmonicida* in coho salmon and rainbow trout.

Lipopolysaccharides (LPS)

LPS is the major component of gram negative bacteria cell wall. The immunostimulatory effects of LPS have been demonstrated in fish [8, 9] and shrimp [10, 11]. LPS can stimulate B cell proliferation and enhance macrophage phagocytic activity [9]. LPS at low doses increase disease resistance and acts as a prophylactic agent [12]. It is demonstrated that shrimp fed with LPS-coated feed provides significantly higher survival rate when challenged with *Vibrio harveyi* [13]. In fish, LPS stimulates the production of macrophage activating factor and the production of interleukin 1 like molecules in goldfish and catfish [14].

Freund's complete adjuvant (FCA)

FCA is a mineral oil adjuvant containing killed or inactivated *Mycobacterium butyricum*. FCA showed increases in respiratory burst, phagocytic and NK cell activity [15]. Injection with FCA has shown increased disease resistance against *A. salominicida*, *A. hydrophila, V. ordalli, Furunculosis*, Red mouth disease and vibriosis [4] in fresh water fishes.

Vibrio bacterin

Vibrio bacterin is the bacterial derivative that has produced increased protections against vibrio infection in fishes and also exhibited immunostimulatory effects in shrimp. [4]

Glucan

Glucan (peptide-glucan-ß-1, 3, glucan) showed immunostimulatory effect by enhancing the lysozyme and macrophage activity. It also offered increased protection in fishes against *V.anguillarum*, *V. salmonicida* and furunculosis. [4].

Animal Sources

Chitin

Chitin is the most abundant polysaccharide in nature and a common constituent of insect and crustacean exoskeleton and fungal cell wall. Chitin is reported to provoke the defence mechanism in a very short time by enhancing the macrophage activities, haemolytic complement activity, leukocyte respiratory burst activity and cytotoxicity. In fishes chitin showed an increased resistance to *A. salmonicida, V. anguillarum* and *P. piscicida* in brook trout, rainbow trout and yellowtail respectively (4 - 23). Chitin supplementation had stimulating effect on the growth and survival of *Macrobrachium rosenbergii*[16]. In shrimps, chitin has been used as an immunostimulant and was known to improve the disease resistance against *V. alginolyticus*[17]

Chitosan

Chitosan is a linear homopolymer of ß-(1, 4)-2-amino-deoxy-D-glucose and is prepared by the alkaline deacetylation of chitin obtained from crab shell. Chitosan has been used as an immunostimulant for increased protection against bacterial diseases in fish and shrimps [4]. Chitosan treatment enhances the phagocytic activity, NBT, Myeloperoxidase, lysozyme activity, neutrophil activity and Ig concentration [18].

Fermented products of chicken egg (EF203) extracts

Fermented products of chicken egg (EF203) contain immunoactive peptides which showed immunomodulatory effects by enhancing the activity of phagocytes and increasing the resistance against bacterial infection [7].

Extracts from Invertebrates

Animal extracts derived from some invertebrates have immunomodulatory effects. An extract from marine tunicate *Ecteinascida turbinata* (Ete) and glycoprotein fraction of water extract from abalone *Haliotis discus hannai* (Hde) reported to be having immunostimulatory effects by showing antitumor activity, enhanced activity of phagocytes and NK cells [4] and also showing increased survival against bacterial infection [4].

The heat extract from firefly squid, *Watasenia scintillans*, show immunostimulatory effects by stimulating the production of superoxide anion, potential killing activities by macrophages and the lymphoblastic transformation of lymphocytes[19].

Glycyrrhizin

Glycyrrhizin is a glycosylated saponin, containing one molecule of glycyrretinic acid, has shown immune enhancement effects by enhancing the respiratory burst activity and lymphocytes activity in both fish and shrimp (20- 4, 4- 23) and also having anti-tumor activities [21].

Nutritional factors

Vitamin C

Vitamin C is essential for the normal growth and physiological process of fish [22] and shrimp [23]. Apart from that it has reported to act as an immunostimulatory agent, by activating the phagocytic cells, myeloperoxidase content and inactivating the free radicals produced by normal cellular activity and various stressors.

Vitamin E

Vitamin E enhances both specific and cell mediated immunity [24]. Vitamin E deficiency will result in reduced growth, muscular dystrophy,anaemia and reduced protection against infection.

Carotenoids

In 1993, Chew first established the role of carotenoids in the immune response of animals [25]. Dietary carotenoids in combination with Vitamin A, C, and E were found to enhance the complement, lysozyme, and phagocytic activities in fish [26]. In shrimp supplementation of carotenoids showed increased resistance to stress, salinity shock and improved the antioxidant response [23, 27].

Trace elements

Dietary supplementation of trace elements such as Zn, Se, Cu improved the immune response, growth and protection against infectious diseases in both fish and shrimp [28].

Hormones

Growth hormone (GH)

Growth hormone (somatotropin or somatropin) is a peptide hormone that stimulates growth and cell reproduction in animals. Growth hormone also acts as an immunostimulant in fishes [28]. In shrimp larvae, recombinant bovine growth hormone was found to enhance growth and immunity [28].

Lactoferrin (LF)

Lactoferrin in is a glycoprotein that influences the proliferation of various pathogens such as bacteria, viruses, fungi, protozoa and exhibit antimicrobial activities. Dietary administration of bovine lactoferrin offered enhanced immune activity in crustaceans [28].

Nucleic acids

(i) Dietary Nucleotides

Nucleotides are the building blocks of DNA and RNA and play vital roles in various physiological and biochemicals functions of the body. Dietary nucleotides are reported to act as immunostimulatory agents by showing increased resistance against stress and increased resistance towards pathogens in salmonids [29]. Recently, nucleotides has received increased attention as immunostimulators.

(ii) CpG Oligodeoxynucleotides (CpG ODN)

CpG Oligodeoxynucleotides are short, single-stranded synthetic DNA molecules comprising cytosine triphosphate deoxy nucleotide "C" followed by a guanine triphosphate deoxynucleotide "G" with phosphodiester "p" linking the two nucleotides. CpG motifs are considered pathogen-associated molecular patterns (PAMPs). CpG motifs are more prevalent in bacterial DNA, and when un-methylated, function as immunostimulants [28].

Application of immunostimulants

For the effective use of immunostimulants, the timing, dosages, method of administration and health status of the animal needs to be taken into consideration.

Timing

The application of immunostimulants should be before the outbreak of disease to reduce the disease-related loss [30]. Immunostimulants can stimulate the defence mechanism from the immunosuppression state caused by stress.

Route of administration

The outcome of using an immunostimulant is usually determined by the method/ route of administration. Immunostimulants can be administered through injection, immersion or oral administration. The injection route may be the most cost-ffective method for the larger fish/shrimp. Immersion method may be the most cost-effective in smaller shrimp/fish. However, these methods are laborious, time consuming and stressful. Oral immunostimulant is a non-stressful method that can be used with fish of any size but requires a high dose of the immunostimulant. Both oral and immersion methods have shown successful enhancement /stimulation of the innate immune system against pathogens, still oral administration is the most practical way of approach [23].

Dosage

The effect of the immunostimulant is mainly determined by its right dosage. In certain cases, higher dose may not offer more protection or stimulation of the immune system. Higher dose of levamisole and glucan may suppress the immune response and low doses may not be effective at all [19]. The effect of the immunostimulant is not directly dose dependent; higher doses may not enhance and may inhibit the immune system [4].

Conclusion

In aquaculture, the effective approach to overcome the disease problem can be attained through better management practices. As proper management is always not delivered, pathogens become established in animals and produce disease. Vaccines, chemotherapeutics and immunostimulants are employed to combat the epidemics of diseases.Vaccination is the most reliable method, but there are no effective vaccines against most viral diseases. Chemotherapeutants are effective in controlling the disease that had already occurred or during the outbreak, but they are expensive and have negative impacts such as residue accumulation, drug resistance and immunosuppression. Immunostimulants may be able to compensate the limitations of both vaccines and chemotherapeutics. Application of immunostimulants is thought to be safer than chemotherapeutics and the range of their efficacy is wider than that of vaccination. Further, a combination of the immunostimulants with vaccines increases the potency of vaccines and the combination of immunostimulant, probiotic, antimicrobial proteins and manipulation of environment may be of much use during disease outbreak. Hence, in the application of immunostimulants, multidimensional comprehensive approach would be much helpful in controlling the epizootics of diseases.

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Plant-based immunostimulants in aquaculture

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Introduction

Aquaculture is the fastest growing animal food producing sector in the world with rapid expansion up to 15 per cent of the internationally traded seafood products[1]. However, world aquaculture production is vulnerable to emerging disease outbreaks due to culture intensification, resulting in partial or total loss of production[2]. Outbreaks of infectious and non-infectious diseases cause heavy loss to the farmers. Increasing the production per unit with increase in the stocking density leads to poor water quality and underprivileged physiological environment. The immunosuppression caused by stressful environment predisposes the fish and shellfish to various infections. Those infections may be controlled either by sanitary prophylaxis, disinfection or chemotherapy. But, application of any chemicals and antibiotics in aquaculture is neither desirable nor economical since it leads to development of microbial antibiotic and chemical resistance and consumer reluctance. Hence, more attention has been paid for the use of preventive methods such as vaccines and immunostimulants both in finfish and shellfish aquaculture. Use of the immunostimulants such as glucan, chitin, lactoferrin, levamisole and some medicinal plant products is advisable to control fish and shellfish diseases; among which the natural plant products are most preferred. Plants and their byproducts contain several alkaloid, lectin, phenolic, polyphenolic, quinone, polypeptide and terpenoid compounds. They are very effective alternatives to antibiotics, vaccines, chemicals and other synthetic compounds without posing any environmental hazard. Both innate and adaptive immunities are enhanced in fish and shrimp by administration of plant based immunostimulants at various concentrations either *per os* or parenteral route against bacterial, viral and parasitic diseases. This chapter is aimed at describing in detail about the plant-based immunostimulants and their application in fish and shrimp aquaculture.

Immunity in fish and shellfish

The immune system in fish, like in other vertebrates, is classified into innate (non-specific, first line of defence) and adaptive (specific or acquired by immunological memory). The adaptive immunity is further classified into natural and artificial. The natural adaptive immunity may be either passive from the maternal antibodies or active by antibody production against specific pathogen. Likewise, the artificial adaptive immunity may be either passive by antibody transfer or active by immunization [vaccination] [3,4].

Unlike in fish, innate immunity is the only immune mechanism available in shellfish. The major components of the innate immune system in fish are neutrophil, macrophages, monocytes, granulocytes and humoral elements, including lysozyme or complement system[5]. The innate immune system in shellfish is by humoral (cell free molecules in the haemolymph) and cellular (haemocytes) components. Based on the cytoplasmic granules, the circulating haemocytes are classified into agranular or hyaline, semigranular (with small granules) and granular (with large granules) cells. They participate in wound and shell repair, nutrient transport, digestion and excretion processes. Hyaline haemocytes involve mainly in coagulation processes while granular haemocytes perform phagocytosis, encapsulation and regulation of the prophenoloxidase (proPO) system. The lymphoid organ (LO) is responsible for filtration and elimination of bacteria. The humoral immune response in shellfish includes melanisation and clotting cascades, anti-oxidant defense enzymes like superoxide dismutase, peroxidase, catalase and nitric oxide synthase, defensive enzymes like lysozyme, acid phosphatase and alkaline phosphatase, reactive oxygen and nitrogen intermediates, and antimicrobial peptides[6].

Plant-based immunostimulant

Prophylactic use of antibiotics and chemotherapeutics has negative impacts such as immunosuppression, development of resistance, and bioaccumulation in the tissues and environment[7]. Hence, strengthening the defence mechanism of fish and shrimp through prophylactic administration of plant-based immunostimulants is considered as a promising alternative. Natural plant products with active principles such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids, and essential oils are identified for modulation of the innate immune, anti-stress, growth promotion, appetizer, immunostimulation, aphrodisiac and antimicrobial properties in fish and shrimp[8,9]. But, the lack of scientific mechanism of action and standardization hamper the wide acceptance of herbal immunostimulants not only in aquaculture but also in veterinary and human medicine[10]. The active herbal compounds may inhibit or block the transcription of the virus to reduce the replication in the host cells and enhance the innate immunity[11]. Ethanol extract with polyvinylpyrolidone (PVP) from *Clinacanthus nutans*-enriched diet protects shrimp from yellow head virus (YHV) infection[12]. *Azadirachta indica*[13, 14] and *Allium sativum*[15] extracts have been reported to enhance the innate immunity in fishes. Seaweed extracts and butanolic extract of *Withania somnifera*[16] are found to protect shrimp from *Vibrio parahaemolyticus* and *V. damsela* infection. In addition, there are also a number of other plants found to have immunomodulating effect on fish and shrimp as shown in the table 1.

Table 1: List of plant immunostimulants with innate and adaptive immune response in fish and shellfish

Plants	Immunostimulation
Achyranthes aspera	An, Ba, He, Ly, SOD[17]
Azadirachta indica	Co, Ly, Pa, RBA[13,14]
Allium sativum	An, Ba, Ly, Pa, SOD[15]
Aloe vera	Ly[18]
Cinnamomum kanehirae	Pa, PO, RBA[19, 20]
C. verum	Ly, Pa, RBA[21]
Eclipta alba	An, Co, Ly, Me, RNS, ROS[22]
Gelidium amansii	Pa, PO, RBA, SOD[23]
Gracilaria fisheri	SOD, THC[24]
G. tenuistipitata	Pa, PO, RBA, SOD, THC[25, 26]
Laurus nobilis	Ly, Pa, RBA[27]
Nyctanthes arbortristis	Co, Ly, Me, RNS, ROS[28]
Ocimum sanctum	An, Nu[29]
Porphyridium cruentum	RBA[30]
Punica granatum	Co, Ly, Pa, RBA[31]
Panax ginseng	GPx, SOD[32]
Sargassum duplicatum	Pa, PO, RBA[33]
S. fusiforme	PO, SOD[34]
Solanum trilobatum	Ly, RNS, ROS[35]
Tinospora cordifolia	An, Co, Ly, Me, RNS, ROS[36]
Toona sinensis	Ig, LY, Pa, RBA[37, 38]
Viscum album	Ly, Pa, RBA[39]
Withania somnifera	Ig, NBT, Pa[40]
An: Antiprotease, Ba: Bactericidal, Co: Complement, GPx: Glutathione peroxidase, He: Hemagglutination, Ig: Antibody, Ly: Lysozyme, Me: Meloperoxidase, NBT: Nitroblue tetrazolium test, NO: Nitric oxide, Nu: Neutrophil, Pa: Phagocytosis, PO: Phenoloxidase, RBA: Respiratory burst activity, RNS: Reactive nitrogen species, ROS: Reactive oxygen species, THC: Total haemocyte count.	

Polysaccharides from various traditional medicinal herbs have been shown to be immunopotentiating both *in vivo* as well as *in vitro*[32, 34]. Natural immunostimulants are biocompatible, biodegradable, cost effective, and safe for the environment. In contrast to

vaccines, immunostimulants enhance the innate immune response[9]. Hence, application of immunostimulants in combination with vaccine boosts immunity in fish and shrimp, thereby the small dose of vaccine is enough to get desirable immunity[41]. These immunostimulants could ameliorate the immune status by encouraging the phagocytosis, bactericidal activities, lysozyme activities in fish and shellfish, and stimulating the natural killer cells, complement system and antibody responses in fish. The effects of the immunostimulant may be species specific. Hence, detailed studies are required for each immunostimulant to elicit dose-response relationship, threshold dose (benchmark dose), biologically effective dose and therapeutic window. Several authors have reported that immunostimulation also has growth-promoting activity[42].

Administration

Immunostimulants are usually administered as immunoprophylactics which potentiate the immunity of the host to protect against pathogens. They are commonly administrated *per os* (diet), bathing or parenteral (intra-peritoneal or intramuscular) [9,31,41]. The route of administration has differential effects on the immune system. Although intra-peritoneal injection has been proved to be the most rapid and effective way of administration, it is labour intensive and relatively time-consuming. It becomes impractical and stressful to fish when fishes weigh less than 15 g. Hence, incorporation in the diet is regarded as the most suitable for fish farming, the method being non-stressful and permits a larger number of fish to be treated with the minimum cost and effort[43]. The effects of immunostimulants for aquaculture depend on various factors like time, dose, method of administration, and the physiological condition and life stage of the fish. The innate immune parameters such as lysozyme, complement, antiprotease, meloperoxidase, reactive oxygen species, reactive nitrogen species, phagocytosis, respiratory burst activity, nitric oxide, total haemocytes, phenoloxidase, glutathione peroxidase, and phenoloxidase, and adaptive immune parameters such as antibody titre, bactericidal, hemagglutination, neutrophil against bacterial, fungal, viral, and parasitic diseases in different fish and shellfish are enhanced by immunostimulants.

Conclusions

Increasing trends of disease outbreaks in intensive aquaculture signify the need of therapeutics. At the same time, the therapeutics should be environment friendly. Hence, research on using herbs as therapeutics, prophylactics and immunostimulants to control diseases in aquatic animals is the need of the hour. Herbs are inexpensive, biodegradable, environment friendly, immunostimulants, therapeutics, and locally available with a broad spectrum of activity against all pathogens. Herbal immunostimulants can be supplemented or supplanted with vaccines, antibiotics and chemotherapeutics in aquaculture. Commercial

vaccines are too expensive, effective against only one pathogen and have bottlenecks in application strategy. But, disease outbreaks are frequently associated with immune status of fish and shrimp. Most of the pathogens are opportunistic and cause disease by taking advantage of the immunocompromised status or stressed conditions of fish and shrimp. Hence, herbal immunostimulants can be used as an alternative solution to maximize fish/shrimp immunity, thereby making them robust to avoid or face diseases in aquaculture.

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Nanoparticle-based immunoprophylactics

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Introduction

In the recent years, aquaculture has taken centre stage as a promising sector fulfilling nutritional security, employment generation, livelihood and also a commercial entity. However, emergence of diseases has rendered the sector a risky venture. A reliable and safe measure to control diseases is necessary. Disinfectants and antibiotics have been considered as a general prophylactic/ therapeutic strategy to contain bacterial/viral/ fungal infection. Unscientific application of such drugs or chemicals is known to cause ecological imbalance and may aid in development of resistant strains. Application of antibiotics below the minimum inhibitory concentration or improper exposure period will cause development of antibiotic resistant bacterial strains. In such scenario, alternative measures to contain pathogens in aquaculture are essential [1- 3]. In addition, water being universal solvent, application of drug in aquatic media is a challenge. A good carrier for delivery of the drug will significantly enhance the efficacy of the treatment. In such a scenario, nanotechnology for prophylactic/ therapeutic purpose can be promising. Nanotechnology is the science where materials of nano-scale (<1-100 nm) are designed, developed and applied. The ultra-small size of the particles gives higher surface to the volume ratio and can pass through biological membranes for effective performance [4]. Bacteria can develop resistance against antibiotics but not against nanoparticles (NP) because unlike antibiotics, NPs exhibit multiple simultaneous antimicrobial properties. Since bacteria cannot develop multiple mutations to acquire resistance against particular NP, there are no chances of development of resistance against NPs [2]. The NPs that have multiple antimicrobial activities are chitosan NPs, metal NPs, and nitric oxide releasing NPs (NO-NPs).

Different nanoparticle forms are being used as nanomedicine. The carrier NPs may carry the drug on its surface or the NPs may cover and protect drug at the core [5]. Liposomes that are lipid bilayer nanoparticles, resembles eukaryotic cell membrane, can be used for delivery of lipophilic and hydrophilic drugs [6]. NPs can be used as carriers of drug, vaccine, genes for prophylactic or therapeutic purposes, as antimicrobial agents or for diagnostic applications [7]. The NPs that have potential as prophylactic/therapeutic application in aquaculture are discussed in brief.

Prophylactic nanoparticles

Nanoparticles that simultaneously show multiple antimicrobial mechanisms give no chance of development of resistant strain. Such NPs have added advantages in

aquaculture as potential agents of prophylactic measure and the most important among them are discussed in here.

a. Nitric oxide releasing NPs: Nitric oxide (NO) is part of host innate immune defence mechanism. Once presence of a pathogen is detected, macrophages like phagocytic cells induce production of NO. Nitric oxide can damage microbial DNA and activate several host innate immune pathways to kill the pathogen [8]. NOs react with thiols to produce s-nitrothiol that can inhibit spores of bacteria [9]. In addition, NO NPs aid in wound healing [10,11]. Nitric oxide releasing NPs such as Mesoporous silica nanoparticles, nitrite containing hydrogel/glass composites etc are being used as nanomedicines [12, 13]. However their application in aquaculture is limited.

b. Chitosan Nanoparticles: Chitosan is a biopolymer derived from chitin, a structural polysaccharide from crustacean exoskeleton. Chitosan NPs act as a good antimicrobial agent. These NPs are polycationic in nature and hence effectively binds with negatively charged bacterial cell wall and cytoplasmic membranes, causing membrane disruption and lysis of bacteria. In addition, they block protein synthesis by binding to microbial DNA [14]. Chitosan NPs of higher molecular weight show greater affinity towards Gram positive bacteria and *vice-versa* [15]. Chitosan's polycationic nature has high affinity towards metallic compounds, hence chelates metalloproteins of bacteria (2). Furthermore, it becomes an ideal carrier system and platform stabilizer for delivery of various NPs (14).

c. Metallic nanoparticles: Metals containing NPs, such as silver, gold or copper, have been used for treatment for centuries. Most famous among them are the NPs of silver, gold, copper, zinc, magnesium and titanium. In fishes and shellfishes, uptake of metal oxide NPs is through gut or gills. Attachment of these NPs on gill mucosa may cause oxidative stress. However, with present knowledge, there are no adverse acute health issues on aquatic biota due to metal oxide NPs, provided they are applied for prophylactic/therapeutic purposes [15,16].

   i. *Silver nanoparticles*: Silver ion (Ag+) is the active constituent that acts against microbes. When silver is added in water, Ag+ ions are formed that bind with sulphur and/or phosphorous containing proteins in bacterial cell wall or cell membrane and kill the bacteria. Furthermore, once Ag NPs make pores and enter into the bacterial cell, it blocks electron transport chain, damage bacterial nucleic acids and denature 30s ribosomal protein. The efficacy of Ag NPs is higher if the size of the particles is <10 nm or truncated NPs. Since Ag NPs show various antimicrobial properties, acquiring resistance against these NPs is very tough [2, 7,14].

ii. *Zinc oxide nanoparticles*: ZnO NPs show versatile antimicrobial properties. The NPs bind lipid and proteins of bacterial cell membrane to break them and kill the bacteria. They release reactive oxygen species to kill bacteria. Coating of polyvinyl alcohol significantly enhances the membrane permeability of ZnO NPs to induce oxidative stress [2, 7, 14].

iii. *Gold nanoparticles*: Au-NPs exhibit very low toxicity to eukaryotic cells, compared to other NPs, Au-NPs are less effective as antimicrobial agent. Au NPs with antibiotics, zeolite showed higher antibacterial property [2, 7].

iv. *Copper nanoparticles*: Cu binds with amine and carboxyl group of bacterial cell surface to impart antimicrobial action. Cu NPs are cheaper and quickly mix with polymers than Ag NPs. Though Cu NPs are weaker than Ag NPs as antibacterial agent, they have a broader range of activity against bacterial and fungal pathogens [2, 7, 14].

Immunomodulatory nanoparticles as adjuvants and in vaccine delivery

Nanoparticles can also be applied as carrier of drug/vaccine in oral treatment. They will protect the content and release at the desired site for higher efficacy. The carrier NPs may be natural such as chitosan or synthetic such as poly ε-caprolactone (PCL) polymer, polymethyl methyl methacrylate (PMMA). PCL coated over the drug can be used in oral treatment. It protects the inner core from digestive degradation in stomach but releases in the intestine [17]. On the other hand, PMMA performs additional immunomodulatory role to enhance antigen presentation and higher antibody titre [14, 18]. Chitosan is an excellent natural drug delivery vehicle. In fishes, chitosan has been used as delivery system for vaccination, hormone treatment and nutritional administration. With its immunostimulatory role, chitosan NPs aid in improved vaccine efficiency. Chitsan NPs have been effective to deliver vaccine against infectious salmon anaemia virus (ISAV) in salmon and trouts, *Vibrio anguillarum* vaccine for Asian seabass (*Lates calcarifer*) and *V. parahaemolyticus* vaccine for black seabream (*Acanthopagrus schlegelii*) [7].

Nanoparticles in disease diagnosis

Disease diagnosis by using nanotechnology can be termed as nanodiagnosis. Gold NP is most commonly used for diagnostic purpose. One of such is Au-NP used for immunodiagnosis of furunculosis in salmons. Loop-mediated isothermal amplification (LAMP) combined with Au NPs increased the sensitivity of the diagnosis of shrimp diseases, white spot syndrome virus (WSSV), yellow head virus (YHV) etc. The use of magnetic nanoparticles coated with secondary antibody in immunomagnetic reduction assay for diagnosing nervous necrosis virus improved the efficiency and sensitivity of the diagnosis. Virus titre can be determined by the magnetic immunoassay analyser [7, 20-22].

Conclusion

Nanotechnology has provided a new window of opportunities in prophylaxis, therapeutics and disease diagnosis. The advantage of nanoparticles is their high surface area to volume ratio, making them highly reactive. In addition, multiple anti-microbial activities make them highly efficient, surpassing development of resistant varieties. They allow incorporation of other nanoparticles, antimicrobials, vaccines, drugs etc., hence acting as an efficient carrier vehicle to the site of delivery. In addition to their antimicrobial properties, they also act as immunomodulators, enhancing innate immunity, antigen presentation aiding strong adaptive immunity. Thus application of nanotechnology in aquaculture will support the establishment of an environment-friendly sustainable development of the sector.

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Biomolecules for prophylaxis in finfish and shellfish
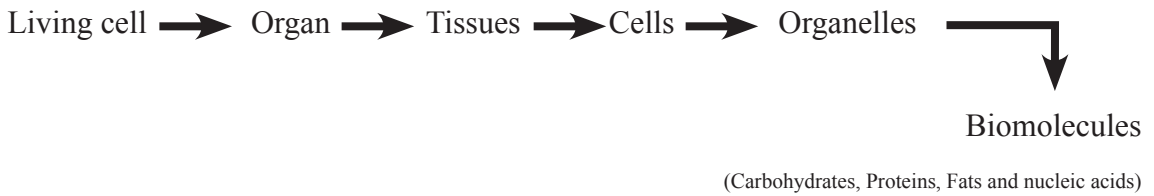
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Introduction

Aquaculture remains an important food and income source for many people globally. According to recent FAO reports, fish accounted for about 17 per cent of the global population's intake of animal protein and 6.7 per cent of all protein consumed. Cultured finfish and shellfish account for 25% of world aquatic animal production. The aquaculture of crustaceans as well as fin fishes is faced with an alarming rise in diseases /threatening the industry's sustainability. Further, it is of worldwide concern, since the economic development of many countries with varying climatic conditions has been affected. The disease development in shell fish and fin fish aquaculture resulted not only from an intensification of production, but also from deterioration in the culture environment, ecological and environmental disturbances and nutritional imbalances. These are all found to affect the aquatic animal's immune system in a negative way. Hence, there is a need to strengthen the aquatic ecosystem governance to deal with the increasing use of water space and resources. Sustainable aquaculture may be ensured with the exploration of modest use of biomolecules in aquatic animals.

Definition

Biomolecules are complex organic molecules that are generally formed by a living organism. They are the molecules involved in the maintenance and metabolic processes of living organisms. They are organic molecules and mainly include macromolecules like carbohydrates, proteins, nucleic acids, lipids and polysaccharides and micromolecules such as natural products, primary and secondary metabolites. The carbon compounds we get from living tissues can also be called biomolecules. They are also known as biogenic substances. They are absolutely present in the living organisms. They are formed in the body by biological means and manage the physiology and growth. Biomolecules are present in the body of humans, animals and plants. Their primary formation from the basic elements seems to occur in plants. Once formed, these molecules pass on to animals through the food chain. Their reactions involve complex mechanisms. Biomolecules are related to living organisms in the following sequence:

Living cell ⟶ Organ ⟶ Tissues ⟶ Cells ⟶ Organelles ⟶

Biomolecules

(Carbohydrates, Proteins, Fats and nucleic acids)

Types of Biomolecules

There are several types of biomolecules. The most important are the nucleotides that make up DNA and RNA, the molecules that are involved in heredity. There are also lipids which function as the building blocks of biological membranes and as energy providing molecules. The carbohydrates are important as energy storage molecules. Amino acids and proteins function in many capacities in living organisms including the synthesis of proteins, in the genetic code and as biomolecules that assist in other processes such as lipid transport. Vitamins are necessary for the survival and health of organisms and though not synthesized by organisms they are important biomolecules.

A diverse range of biomolecules exist, including

Small molecules: Lipids, phospholipids, glycolipids, sterols, glycerolipids, carbohydrates, sugars, vitamins, hormones, neurotransmitters, metabolites

Monomers: Amino acids, nucleotides, monosaccharides

Polymers: Peptides, oligopeptides, polypeptides, proteins, nucleic acids (DNA, RNA), oligosaccharides, polysaccharides (including cellulose), lignin and hemoglobin

Small Molecules – Sugars as an immune biomolecule

In aquaculture, beta-glucan and its derivatives have been used often and have resulted in increased success in enhancing non- specific immunity against various diseases. It is a polymer of glucose known as glucan, which is present in the cell walls of fungi, plants and bacteria, it is a potent immunostimulant in fish and shrimps [4]. However, it has to be used at an ideal level. High dose was capable of causing negative effect on animal survival as well as pathogen protection; hence an optimal dose is recommended [15]. Further, it was found to control vibriosis also [9]. They are capable of increasing shrimps' immunity up to four weeks and protect at from the white spot syndrome virus (WSSV) [2]. Commercial products derived from h-glucans, polysaccharides, yeast or bacterial components have been shown to increase the immune reactions of the animals and, therefore, their capability to eliminate pathogens [19]. In other respects, it has been demonstrated that, upon stimulation by h-glucans and polysaccharides, haemocyte exocytosis is activated, leading to the release of effectors belonging to the proPO system, which can contribute to eliminate microorganisms [17].

Monomer - Amino acid as an immune biomolecule

Amino acids are prerequisite for the synthesis of a variety of specific proteins (including cytokines and antibodies) and regulate key metabolic pathways of the immune response to infectious pathogens. Acceptable dietary inclusion of all amino acids is obligatory for sustaining normal immuno-competence and protecting the host from a variety of diseases in all species. Because of a negative impact of amino acid imbalance and antagonism on nutrient intake and utilisation, an excess supply of amino acids in the diet can be deleterious to the immune system. Thus, care should be taken in developing effective strategies of enteral or parenteral supplementation to achieve maximum health benefits.

Polymer - Antimicrobial peptides as a promising immune biomolecule

Anti-microbial peptides are defined as anti-microbial agents made by an animal, including humans, with a function that is important for the innate immunity of that animal. Antimicrobial peptides are ubiquitous, and are found in both prokaryotic and eukaryotic organisms. These compounds are classified into five distinct groups based on amino acid sequences, secondary structures, and functional similarities. About 400 antimicrobial peptides have been isolated, mainly (about 50%) from insects, but also from plants and vertebrates. They share common features, have a high specific function and are generally non-toxic to eukaryotic cells. They are active against a broad range of microorganisms, including Gram-positive and Gram-negative bacteria, fungi, yeast and, in some cases, viruses and protozoa [10, 13,14]. Most of the peptides act directly on the microbial membrane, interfering with its permeability properties or by pore-forming action. Some interfere with microbial membrane biosynthesis causing cell death (attacins) [5]. In crustaceans, antimicrobial peptides and polypeptides have been isolated from the haemocytes of the crabs. Recently, a polypeptide has been characterized from the haemocytes of shrimps *L. vannamei* and *L. setiferus* [8]. Similarly, from *P. monodon* [18], homologies to the Limulidae anti-LPS factor (anti-lipopolysaccharide factor) which has a strong antibacterial effect, especially on the growth of Gram-negative bacteria was found. A family of antimicrobial peptides, named penaeidins, has been fully characterized from the haemocytes of *L. vannamei* [6] and recent results show that these peptides are ubiquitous in crustaceans. The penaeidins display antifungal and anti-Gram-positive bacterial activities and are synthesized and stored in cytoplasmic granules of the circulating haemocytes of shrimp. The dual functions of penaeidin (chitin-binding property and antimicrobial activity) are important in chitin assembly, wound healing and in protection of shrimp during developmental and moulting stages. Besides penaeidins, crustaceans have several biologically active molecules like agglutinins, killing factors, lysins, precipitins, cytokine-like molecules, clotting agents which influence the immunity. However, further fundamental research is necessary to establish the real effect of such products.

In fin fishes, a number of substances are capable of inhibiting bacterial growth and one such molecule is transferrin. Iron is an essential element in the establishment of infection by most pathogens but the availability of iron in vertebrate tissue fluids is extremely low because it is bound with high affinity to the blood protein transferrin. Pathogenic bacteria have evolved several mechanisms to obtain iron from the host including that from transferrin. However, transferrin exhibits a high degree of genetic polymorphism, and the ability of certain bacterial pathogens to obtain iron from transferrin may be restricted to certain host transferrin genotypes [1]. Besides this, fish plasma contains a number of protease inhibitors, principally α 1-antiproteinase and α 2-macroglobulin. Lectins are other proteins capable of inhibiting the growth of certain bacteria in fishes. Lysins, an anti-bacterial peptide, has the ability to disrupt bacterial membranes and have been reported from fishes. Recently, a number of antibacterial peptides have been isolated from the skin secretion of fish species. The skin mucus of many fish species contains trypsin-like activity, lysozyme. C- reactive protein is seen in fish serum, reacts with phosphorylcholine which is seen on the surface of bacteria, fungi and parasites capable of activating complement and thereby activates lytic and phagocytic defences. Besides, fishes also produce certain anti-adhesive substances, anti-toxins, anti-invasins and complement [1]. Hepcidin, another antimicrobial peptide and an important mediator of the immune response against bacteria in fishes, helps in iron regulation and the innate immune response. The teleost immune system has many of the key molecules needed for control of diseases like a large number of cytokines and cell surface-associated receptor ligand systems that mediate co-stimulatory elects [7].

Approaches for the control of diseases in aquaculture

Diseases occur due to the commotion of the equilibrium existing between the animals, the environment and the pathogens [16]. Present day aquaculture industry needs to be reviewed regarding the control of diseases and adapted health management. A multidisciplinary approach is essential to tackle it. In short, much more basic knowledge on physiology and genetics of the cultured species is required. The aquaculture farmers have been using wide range of chemicals such as disinfectants, vitamins and antibiotics to treat water as well as animal as preventive and curative measures [3]. This has led to the development of drug-resistant bacteria [11] and environmental imbalances [12]. Recently many prophylactic measures, like the use of probiotic bacteria, have been tried by various aqua-culturists to curtail diseases. Other approaches for improving the health management in aquaculture are now being investigated in order to optimize production by improving responses to stress or to infections.

Conclusion

Many biomolecules show an impressive activity against pathogens in crustaceans and fishes. Hence, due to their biological properties, they may constitute a new type of therapeutic agents with vast potential for application in aquaculture. These natural agents, assayed for their protection against infections, may substitute conventional and chemical drugs, which can be harmful for the environment and for the consumers. It is essential to understand fully the immunodynamics and immunokinetics of these compounds in order to ensure maximum efficiency and effectiveness in their applications. More studies about these compounds or natural substances with beneficial effects are warranted. The challenges of bacterial resistant strains and antibiotic residues could be overcome, paving the way for an eco-friendly aquaculture.

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Immuno-nutrition :

Nutritional prophylactics in aquaculture

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Immuno-nutrition is defined as "the study of enhancing immunological functions by means of using specific nutrients and/or other dietary compounds, which could be higher than those levels needed for optimal growth" [1]. In an aquatic system, there are many pathogens waiting for an opportunity to invade the stressed or malnourished fish. Until recent years, disease outbreaks were managed with the help of antibiotics. It is of common knowledge that antibiotic usage has serious environmental impacts by accumulating in animal tissues and thereby acquiring bacterial resistance [2]. So, in order to avoid the diseases and stress generated due to environmental variation and malnutrition, the immune system of the fish should be strong enough. Hence, it is the need of the hour to ensure that specific and non-specific system of the fish should be stimulated against the common pathogens of the aquatic system. Instead of developing costly vaccines and relying on drugs which cause residual effect, utilization of immunomodulatory effect of nutrients will be a better strategy. Globally, the welfare of farmed fishes is assisted by the enhanced use of immunoceuticals or immune enhancing nutraceuticals [3]. So, different forms of immune formulations are popularised among fish and shrimp growers as a measure of prevention of diseases.

Aquaculture and animal welfare

Aquaculture is an efficient and economical means of protein production from aquatic environment. The last century has witnessed the growth of the global aquaculture sector into a billion dollar industry. During this period, aquaculture practices have aquired several dimensions from an extensive system to highly intensive culture system. However, the intensification of the system alters the physiological conditions and nutritional requirement of the fish [4].This gradually generates stress and brings diseases to the system and it is reported that the current practices negatively affect fish welfare [5]. There are several studies on the effect of stress and its mitigation strategies in aquatic systems [6].The expansion of aquaculture to meet the target of 20mt in 2030 is leading to an increase in fed aquaculture. Hence, the quality of diet is of paramount importance as the efficient utilization of nutrient-rich feed is dependent on it.

Nutrition in aquaculture

Development of artificial feeds are done using different dietary combinations of proteins, carbohydrates, lipids, vitamins and minerals based on the nutritional requirement of the species, its stage of development, size, etc. Diet with balanced nutrients should ensure adequate nutrition and thereby contribute to efficient growth and feed conversions and sustain the normal health. Any alteration or deviation in the nutrient ratios or any negative nutrient interactions may evoke deficiencies, compromising the usual animal health and make the animals susceptible to pathogens [1, 3]. The replacement of conventional feed ingredients with plant-based ingredients reduces the digestibility and enhances the exposure to several antinutritional factors and toxic substances. Moreover, using inappropriate diet brings malnutrition to the animal. So the formulation and preparation of feed is a major factor, controlling the production of a farm. Additionally, stress conditions reduce the feed intake and overall performance of the animal. There are several studies carried out to analyse the immune enhancing properties of various nutrients [3].

Proteins and amino acids

Protein and amino acids are essential regulators of several metabolic pathways in fishes and are required for larval metamorphosis, reproduction, immunity, and resistance to environmental stressors [7]. Amino acid deficiencies are reported to be involved in poor growth in shrimps but not manifested as deficiency signs [8]. Common carp fed with a peptide called apidaecin at 15–30 mg kg−1 improved growth performances and enhanced immune response [9]. A few reports on amino acids in fish and shrimp immunity are detailed in Table.1.

Table 1: Report on aminoacids in fish and shrimp immune response

Amino acid	Function/target	Species	Reference
Arg	Formation of Nitric Oxide	Channel catfish	[10]
Ar, met and gly	Formation of Creatine-antioxidant	Artic charr	[11]
Gln	Fuel for macrophage	Channel catfish	[10]
His	Protection against pH change	Salmon	[12]
Taurine	Osmotic pressure regulation	Carp	[13]
Phe and Tyr	Down-regulated immunity	Shrimp	[14]
Methionine	Optimal growth and survival	Tiger shrimp *P.monodon*	[15]

Lipids and Polyunsaturated fatty acids (PUFA)

Lipids are incorporated in feeds to be used as an energy source and to spare the protein for plastic purposes However, their inclusion level varies and depends on the type of species to be fed [3]. Lipids are also sources of essential fatty acids required by both marine and freshwater fishes [16] for their growth, development and immune functions. Marine fishes have specific n3 FA requirement due to the absence of delta-5 desaturase enzyme for chain elongation [17]. EFA are precursors of eicosanoids and prostaglandin which are involved in immune and inflammatory responses in fishes [16].

Table 2: Role of fatty acid in immune enhancement of fish and shell fish

Fatty Acid	Immune function	Species	Reference
High sunflower oil (rich in n-6 PUFA), low n-3/n-6 FA ratio	Cardiovascular disorders	Atlantic salmon	[18]
Fed 12% or 16% lipids (fish oil/corn oil, 1:1)	Higher plasma lysozyme and alternative complement activities	Groupers	[19]
n-3-HUFAs (arachidonic acid, EPA, DHA	Significantly higher final weight and instantaneous growth rate	*P. vannamei*	[20]
1.5% supplement of purified soya bean phosphatidylcholine (PC)	Significantly improves growth and reduces sensitivity to osmotic stress	*P. vannamei*	[21]

Carbohydrates

Carbohydrate utilisation in fishes is dependent on the species, the type of carbohydrate source, molecular complexity, processing treatments and dietary inclusion level [22]. Carnivorous fishes have the lowest preference for carbohydrate than herbivorous and omnivorous species. While the shrimps have the digestive enzymes required for carbohydrate digestion [23], the juveniles and sub adults have high apparent digestibility of carbohydrate at 40% of starch levels in the feeds [24, 25]. Their enzyme activities depend on the ontogenesis, feeding activity, intermolt period and diet composition [26]. Complex carbohydrates promote growth while simple sugars cause deleterious effect on juvenile shrimps [8]. A few selected reports of carbohydrate on fish immunity are given in Table 3.

Table 3: Role of carbohydrate on fish and shrimp immunity

Carbohydrate	Function	Species	Reference
Dietary chitin	Stimulates the innate immune response by increasing complement activity, cytotoxic activity, respiratory burst and phagocyte activity, but not lysozyme activity.	Gilthead sea bream	[27]
Dietary chitin	Diets interferes with bacteriolytic activity of lysozyme	Trout	[28]

Vitamins

Fishes and crustaceans depend on exogenous sources for vitamins and are to be supplemented in the diets [29]. The deficiencies of vitamins are most common in the fish culture due to their non-availability in the feed or by the antagonistic interactions with other dietary components [30]. The information on the role of vitamins on the immune response is scanty. However certain vitamins (C and E) are strong antioxidants and enhance the immune response when supplemented above their normal dietary requirement. The effect of vitamins in fish immune response is listed in Table 4.

Table 4: Important vitamins involved in immune responses in fish and shellfish

Vitamins	Function	Species	Reference
Vitamin E and n-3 HUFA	Improved non-specific immune responses and disease resistance	Japanese flounder	[31]
Pyridoxine	Improved disease resistance	Chinook salmon	[30]
Folic acid	Improved disease resistance	Channel catfish	[31]
Choline chloride or Ca-pantothenate	Increased complement activity	Red seabream	[32]
Myoinositol	Enhanced non-specific and specific immune responses	Jian carp	[33]
Vitamin A and E	Immediate protection against free radicals	Shrimp	[34]
Vitamin E	Improvement of non-specific immune response	Tiger shrimp	[35]
Vitamin C and E	Potential immunomodulator	Shrimp	[36]

Carotenoids

Carotenoids are lipophilic compounds with high antioxidant activity and even spare vitamin A. Astaxanthin is ten times more powerful antioxidant than other carotenoids. Astaxanthin can cross the blood-brain barrier and bring antioxidant and anti-inflammatory protection to the brain and nervous system. Thomson et al [37] reported that astaxanthin given in rainbow trout diet enhanced immunomodulaory effect. Astaxanthin is required for growth and survival of first-feeding Atlantic salmon [38].

Minerals

Fishes and crustaceans meet their mineral requirement through diets and their direct absorption from the surrounding water [29]. However, certain minerals in the aquatic environment (phosphorus) remain unavailable to animals and needs to be fortified in the diets. In many reports (Table 5) mineral supplementation above optimal level considerably enhance the immune response and disease resistance in fish [39,40].

Table 5: Role of minerals in immune enhancement of fish and shell fish

Minerals	Function	Species	Reference
Fe	Significant increase in catalase activity in head kidney	Atlantic salmon	[41]
Selenium and vitamin E	High intracellular superoxide anion production by macrophages	Channel catfish	[42]
Se	Significantly affected growth and immune response of juveniles, increased antibody production	Channel catfish	[43]
Cr	Modulates immune response by affecting serum lysozyme activity, respiratory burst of head kidney macrophages and phagocytosis by macrophage	Rainbow trout	[44]
Inorganic Selenium	Improves the immune response, disease resistance, and antioxidant status, antimicrobial defense	*Macrobrachium rosenbergii*	[45]

Role of nutrients in larval development

The balance between macro and micronutrients, including amino acids, polyunsaturated fatty acids (PUFA), vitamins and trace elements, performs specific

and essential role in the development of the immune system at the larval stage [46] and maintain optimal health of larvae as well as bigger fish and shrimp. Eicosanoids involved in the regulation of the immune system by their direct effects on cells such as macrophages and lymphocytes or their indirect effects via cytokines [47]. Vitamin deficiencies result in depressed immune function and slow or no recovery from disease in larval developmental stages [46]. Life stage and size of the fishes are also important but age dependent studies of these immunostimulants are lacking. Brood stock diet plays major role in the immunity development of larvae. So the immune stimulation of larvae can also alter through dietary interventions of brooders. However, there is gap in this line. Future research should focus on these issues to increase the immune status of fish.

Conclusion

Nutrients perform specific and essential role in development and maintenance of the immune system at the larval and grow-out stages. However, the use of this information to develop nutritional strategies to strengthen the immune system is still in its infancy. Non-nutritive additives such as vaccines, antibiotics, probiotics, drugs and immunostimulants are widely used in fish and shrimp farms to resist the diseases. However, maintaining the health status of the farming animals through drugs and antibiotics application is expensive and results in bacterial resistance and tissue residues. In this context, defending the pathogens and maintaining health through nutritional manipulation will be a cheaper way of boosting the immune defence in fish and shrimp. The impact of nutrients on the immune system is widely known. Finding the pharmacological dose of each nutrient needs to be undertaken with respect to each species for commercial application.

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Nucleic acid-based immunity

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Introduction

Nucleic acid-based drugs and vaccines contitute a potential substitute line of approach for the prevention of pathogenic microorganisms in aquaculture. The efficacy of nucleic acid-based vaccines is still under dispute among various researchers worldwide. There is need for extensive knowledge and research on immune responses in fish and crustaceans for the development of vaccines and their administration. Vaccination is widely used in mammals and their success in evoking resistance against pathogen were well established with various studies on the immune monitoring design, followed by mass vaccination. The ideal aquaculture vaccination requires effective development of vaccines, proper use in accordance with aquaculture practices like production cycle, water temperature, fish size and revaccination to maintain the immunity level to overcome the infectious gravity of the pathogen. The protection should aim at the highest level of immune response to specific pathogen when there is disease outbreak and it should last throughout the entire period of risk. DNA vaccination is administration of the gene encoding a vaccine antigen. The DNA vaccines mainly interact with the innate immune responses of fish. In shrimp, the use of both exogenous synthetic long dsRNAs or siRNAs have been reported to induce antiviral response.

Immunogenicity of DNA Vaccines

DNA vaccines are plasmid DNA (pDNA) containing a construct for a protective antigen gene capable of being replicated autonomously in prokaryotes. The principle of DNA vaccination is synthesis of polypeptide antigen from the plasmid vector on entry into host cells by transfection on injection of encoded immunogenic protein into the host tissue. The transcribed antigens replicate in the cytosol using the endogenous pathway while soluble or secreted antigens are phagocytized by APC and gain access into the exogenous pathway. The DNA vaccines made for viral diseases are capable of *in vivo* synthesis of antigenic proteins using host cell machinery in a manner identical to natural virus infection. This culminates in antigenic proteins expressed by plasmid DNA gaining access to both the exogenous and endogenous pathways in the activation of both humoral and cellular mediated immune responses. DNA vaccines against rhabdoviruses, such as infectious hematopoitic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV), have been most successful and were found to be effective against hirame rhabdovirus (HIRRV), infectious pancreatic necrosis virus (IPNV), red seabream iridovirus (RSIV),

and spring viraemia of carp virus (SVCV). They may also be useful against intracellular bacterial pathogens, including *Edwardsiella tarda* and *Mycobacterium marinum.*

Tissues involved in Nucleic Acid vaccine uptake

On intramuscular injection of pDNA , a fraction is transferred to the circulatory system and distributed to other organs and tissues. Some are exposed to scavenger receptor in extracellular compartments and the major portion is excreted directly immediately after degradation. pDNA was able to reach organs intact such as kidney, spleen and gills. They can enter scavenger endothelial cells in the thin endothelium layer covering the walls of blood circulatory system which consists of different subpopulation of cells, which are structurally and functionally specialised depending on their anatomical location. The sinusoids of the mammalian liver lobules are lined by fenestrated endothelial cells, with Kupffer cells (fixed macrophages), T lymphocytes, monocytes and natural killer cells, all attached to the luminal side of the endothelial lining. On these liver endothelial cells (LEC), numerous coated pits, vesicles and large amounts of other organelles associated with endocytosis have been detected with pDNA. The liver sinusoidal endothelial cells being a general vertebrate non-phagocytic scavenger endothelial cell (SEC) system, with an extensive capacity to endocytose and degrade soluble physiological and foreign macromolecular waste substances/molecules from the circulation by receptor-mediated endocytosis were also involed in nucleic acid uptake.

Immune cells in taking up DNA vaccines

Fish macrophages involved in the uptake of pDNA are located in several tissues such as the kidney, spleen, thymus, intestine and mesentery. The kidney is the major haematopoietic tissue and is considered to contain the largest reservoir of macrophages. The macrophages are multifunctional cells with phagocytic and secretory properties, and play an important role in host resistance against infections, by killing and digesting invading micro-organisms. In addition, macrophages are professional antigen presenting cells. Phagocytosis is mediated by specific receptors, such as the scavenger receptors, mannose receptors, and various receptors recognising complement factors and the Fc-g receptor. Some of these receptors also mediate effective endocytosis of soluble ligands. The phagocytic nature of macrophages of fish are not as well studied an *in vitro* study of rainbow trout head kidney macrophages suggests that scavenger receptors are involved in phagocytic processes with minor importance in blood clearance of soluble scavenger receptor ligands. Recently studies showed that Atlantic salmon head kidney macrophages are able to take up CpG ODN *in vitro*. Dentritic cells or dendritic-like cells with their cytochemical signatures and function have not yet been characterised in fish. Similarly pDNA has not been detected in hepatocytes and skin cells of fish after i.v. or

i.m. administration. On the other hand, green fluorescent protein (GFP) expression was detected in the epithelial cells of zebra fish skin after gene gun administration of pDNA. Plasmid DNA has been detected in rainbow trout myocytes which are able to express the transgene but the mechanism is not known.

B cell activity and antigen specific humoral immunity were induced by HIRRV G-protein gene. Inoculation of fish with G-protein genes of VHSV and IHNV induced the production of neutralizing antibodies, hence DNA vaccination against VHSV G-protein gene might play an important role in the immune response. DNA vaccination against rhabdovirus provided high levels of specific protection without producing detectable amounts of neutralizing antibodies.

Homing of leukocytes to the injection site suggests that cells expressing the recombinant G-protein had a chemoattractant effect. B lymphocytes, both IgM+ and IgT+ cells, represent one of the major cell types infiltrating the injection sites expressing the G-protein of VHSV. In their study, they showed upregulation of CXCR3B, a receptor for CXCL11, together with CK5B and CK6 chemokines, which could play chemotactic roles in the early recruitment of B-cells at the injection sites.

A cDNA microarray analysis of Japanese flounder immunized with DNA vaccines encoding rhabdovirus G-proteins showed up-regulation of the genes that were involved in cellular immune responses, such as the CD8 α chain gene. The role of cellular immunity in the DNA vaccine-inoculated fish has not yet been clarified. A specific cell-mediated immune response of crucian carp lymphocytes to the MHC-matched cells infected with crucian carp hematopoietic necrosis virus was reported CD8+ cytotoxic T cells (CTLs) were considered to be involved in the antiviral adaptive immunity of the carp. Taken together, CD8+ CTLs of teleosts may also have a role in the specific protection provided by DNA vaccination.

In addition to inducing antigen-specific immune responses, DNA vaccination also induces non-specific innate immune responses. Vaccination of rainbow trout with the G-protein gene from IHNV induced early antiviral defense against a VHSV challenge, suggesting that DNA vaccination is also involved in the activation of the innate immune system. An immediate up-regulation of non-specific immune response genes, including NK Kupffer cell receptors, MIP1-a, and antiviral Mx1 protein (Mx1), following administration of VHSV G-protein genes, was found. Furthermore, the interferon-stimulated gene 15 kDa (ISG15), ISG56, and Mx1 were strongly induced after the vaccination with the HIRRV G-protein gene. Mx1, ISG15, and ISG56 are known as type I interferon (IFN)-inducible genes, which inhibit viral replication and protein synthesis. Similarly, studies had shown type I IFN-related genes were up-regulated at

systemic sites (e.g., the gills, kidney, and spleen) by the IHNV G-protein gene in rainbow trout. Induction of the type I IFN system across multiple tissues may be one of the major functions of early anti-viral innate immunity in DNA-vaccinated fish. For a DNA vaccine to induce an effective immune response against fish pathogenic viruses, there must be both a type I IFN-mediated innate immune response and an adaptive immune response.

The intracellular delivery of the plasmid DNA encoding the recombinant G-protein R of VHSV inside the muscle cells of vaccinated rainbow trout. Intracellular detection of the G-protein was seen up to 45 days at the injection sites. Transcription of the G-protein was demonstrated by detecting of mRNA in muscle tissue extracts, which was linked to expression of high antibody and MHC-II mRNA levels. Activation of the CTLs following immunization using the G-protein of VHSV in rainbow trout was observed, when they used PBLs collected from fish immunized with a DNA vaccine encoding the recombinant G -protein of VHSV and showed that PBLs from vaccinated fish killed the VHSV MHC-I matched RTG-2 cells, indicating that the G-proteins had the capacity to induce CTL responses in vaccinated fish. These studies show that the intracellular expression of proteins transcribed from DNA vaccines in fish cells leads to homing of leukocytes and B-cells to injection sites with possible involvements of chemoattractant chemokines. High expression levels of humoral and cellular responses can be achieved at low doses at nanogram quantities of a DNA vaccine-protected rainbow trout against IHNV infection after challenge.

The immune responses activated by nucleic acid recognition

Pattern recognition receptors (PRRs) are involved in nucleic acid recognition in teleosts. In mammals, the innate immune response is initiated through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). The signaling PRRs include the Toll-like receptors (TLRs), retinoic acid inducible gene (RIG)-I-like receptors (RLRs), and nucleotide-oligomerization domain (NOD)-like receptors (NLRs). The DNA of the vaccine is directly recognized by some of these receptors, which then trigger innate immune responses. These receptors include CpG DNA sensors (e.g., TLR9), B-form DNA sensors (e.g., Z-DNA binding protein-1; ZBP), and inflammasomes. ZBP is also known as DAI (DNA dependent activator of IFN-regulatory factors). Inflammasomes include NACHT-, leucine-rich repeat (LRR)-, and pyrin domain (PYD)-containing proteins (NALP3).

In teleosts, six TLRs and three RLRs have been demonstrated to recognize nucleic acids,PRR-associated sensing of exogenous nucleotides has been shown to lead to antiviral responses via the production of type I IFN and other cytokines. Hence, exogenous nucleic acids (i.e., DNA vaccines, viral RNA, and bacterial DNA) and their analogs can be used as

vaccine adjuvants for aquaculture. TLRs are type-1 trans-membrane glycoproteins, which comprise an N-terminal extracellular leucine-rich repeat (LRR) domain and a C-terminal intracellular Toll/IL-1receptor (TIR) domain. The LRR domain is responsible for the recognition of ligands specific to a particular PAMP. These structural characteristics are conserved in vertebrate TLRs, except the soluble form of TLRs (e.g., TLR5S in teleosts). Several teleost TLRs (TLR3, -7, -8, -9,-21 and -22) may be able to sense nucleic acids.

Conclusion

The benefits of DNA vaccine include both innate and adaptive immune response in fish and is safer than equivalent technologies in many aspects. The main drawback of DNA vaccination is too low immunogenicity with low level expression of transgene in inducing protection against infection. DNA vaccine falls within the definition of a genetically modified medicinal product, hence there are regulatory issues to introduce DNA vaccines in the market.

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