Overview of fish vaccines with focus on nanovaccines

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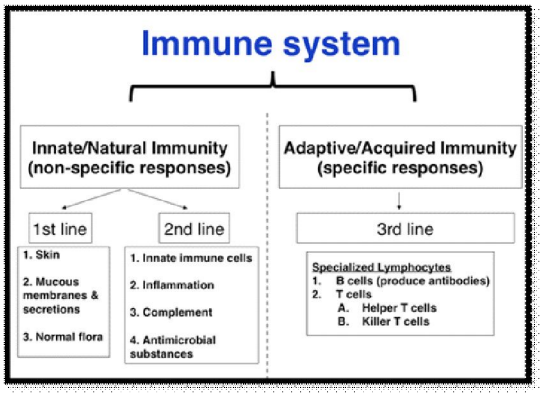
ABSTRACT

The development of a strong aquaculture industry depends, in part, on advances in the diagnosis and treatment of fish diseases. Use of antibiotics has attracted lot of criticism due to the issues like antibiotic residues, bacterial drug resistance and toxicity. In this present scenario, vaccination would be the best alternative to combat bacterial and viral diseasesforlong term protection. The first report on fish vaccination was by David *C. B. Duff and he is regarded as "Father of fish vaccination". In 1942 he reported prolonged use of* chloroform-inactivatedbacteria through feed could protect trout fish from a serious bacterial disease.Vaccination believes in saying "*prevention is better than cure*"& it is a sustainable means of preventing diseases.The major causative agents of infectious diseases in finfish aquaculture include bacteria (54.9 %), viruses (22.6 %), parasites (19.4 %) and fungi (3.1 %)[1].

Traditional vaccines for fish are generally safe but these are often less immunogenic.However, genetic immunization or DNA immunization mediated by plasmid DNA in transfected cellsactsas a bioreactor to produce the vaccine.Some of the advantages of polynucleotide immunization is that it is extremely safe, induces a broad range of immune responses (cellular and humoral responses), long-lived immunity.There is a great need to develop better delivery systems to improve the transfection efficiency in vivo.Recently, biocompatible nanoparticles (NPs) have gained enormous attention as delivery vehicles for vaccines.NPs can be surface-engineered with peptides, proteins, polymers, cell-penetrating peptides, and other targeting ligands, which make them a versatile delivery vehicle for vaccine formulations.



Basis of fish vaccination (the immune response)

In multicellular organisms immunity is necessary for *survivability*to resist harmful microorganisms from entering into body. Immunity involves both specific (acquired or adaptive)and nonspecific (innate or inborn)components. The nonspecific components act as eliminators of a wide range of disease causing organisms or pathogens irrespective of their antigenic make-up.Specific or acquired components, involves special class of cells (T & B-cells) responsible for adaptive immunity& these are targeted to particular pathogens.

Innate mechanisms require no previous exposure to the particular antigen- this includes: physical barriers such as skin and mucus layers, specialized cells such as macrophages and natural killer cells and particular soluble molecules such as complement (a part of the immune system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism) and interferon (group of signaling proteins made and released by host cells in response to the presence of several viruses). The cell-mediated response in fish is similar to that in mammals and relies on the presence of antigen results in a cascade of events that includes cytokine production that regulates or enhances the cellular response.Fish are the most primitive vertebrates to possess adaptive immune system. Adaptive immunity arose early in vertebrate evolution.The innate system is the earliest immune mechanism.Fish have cellular (T mediated) and humoral (B lymphocytes & antibodies mediated) immune responses. Fish and mammals show some similarities and some differences regarding immune function. The two head kidneys (anterior part of trunk kidney) arethe site of formation of blood cellular components(haemopoietic) and also it is the central organ for immune-endocrine interactions.The thymus is situated near the opercular cavity in teleosts which produces T lymphocytes and B cells.They have complement system & cytokines (are low molecular weight, soluble proteins that are produced in response to an antigen and function as chemical messengers for regulating the innate and adaptive immune systems) also as a backup of immune system.Immunomodulatory products, including nucleotides, glucans and probiotics when given as dietary supplementation confer increased resistance to fish against viral, bacterial and parasitic diseases and improves the effectiveness of vaccination and the better ability to osmoregulate. The use of these products reduces the need for therapeutic

treatments, enhances the effects of vaccines.

Three generations of fish vaccines

Attenuated and inactivated vaccines are identified as the FIRST GENERATION, which use a primary method in their production. Attenuated pathogens, full organisms or inactivated bacterial toxin, which are effectively immunogenic, are used in making these vaccines. This type of vaccine is known as a traditional vaccine. Example of this type iskilled vaccines against *Streptococcus* spp. or/ and *Lactococcus* spp. infections in rainbow trout (*Oncorhynchus sp.*).

SECOND-GENERATION vaccines have subunit elements, recombinant or synthetic proteins, non-protein antigens, and epitopes of different species and strains of pathogens.Subunit vaccines are produced by using the part of the DNA which encodes for the production of the specific antigens to trigger an adequate immune response into another type of organism.Subunit vaccines take advantage of using only antigenic components for vaccination and since subunit vaccines cannot replicate in the host, there is no risk of pathogenicity to the host. One commercial subunit vaccine (peptide; VP2) is currently used in Norway (against IPNV).

THIRD GENERATION vaccines have the principles of immunogenic potential administration of a plasmid containing a gene encoding the antigen, known as genetic vaccines& used since the beginning of 1990s. Different names have been given for this kind of vaccine, such as DNA vaccines, RNA vaccines, and plasmid vaccines.In 2005, APEX-IHN (Novartis/Elanco) for protecting Atlantic salmon against Infectious Hematopoietic Necrosis Virus (IHNV) in British Colombia became the first DNA vaccine licensed for commercial use in aquaculture.

Flow diagram (in simplified way)

of immune mechanisms in the vaccinated fish by expression of the VHSV G protein in transfected cell's nucleus harboring the DNA vaccine in plasmid*

Uptake of the vaccine plasmid in the nucleus—-àplasmid promoter will drive transcription of the G gene —-àmRNA transcripts —-à exported to the cytoplasm—-àribosomes binding to the mRNA will mediate translation of the polypeptide into the endoplasmatic reticulum (ER)—-à transmembrane G protein will be folded and glycosylated and associate into non-covalently associated trimers while on its way from ER to the Golgi cisternae—-à reaches the cell membrane in its mature form—-àprotruding as spikes into the extracellular space and appear like a virus infected cell—-àendosomal TLR activates &initiate an IFN type I response—-àactivate intracellular antimicrobial programmes and influence the development of innate and adaptive immune responses—à

*Viral hemorrhagic septicemia virus (VHSV) is the causal agent of a serious disease in many marine and freshwater fish species worldwide.the virus has a negative-sense, single-stranded RNA genome.The VHSV G-protein gene DNA (using reverse genetics) vaccine had a high protective efficiency, giving

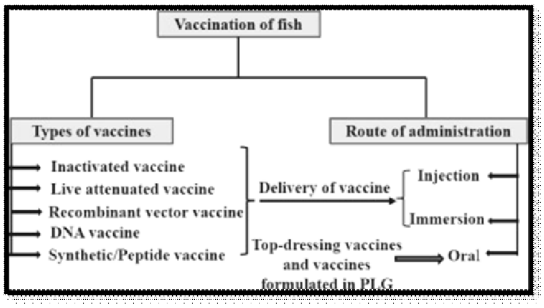
relative percentage survival (RPS) values of at least 93%.VHSV is attenuated using deletion of whole virulent NV gene.Among the components, the glycoprotein (G) is located on the surface of the virus and can induce immune response because of the presence of epitopes.

Concept & use of vaccines in aquaculture

The major goal of vaccination is to induce a specific long-term protection against a certain disease. Among various diseases viral disease outbreak is the most serious issue as it may cause severe losses to the economy in the aquaculture industry worldwide.A typical fish vaccine either contains or produces a substance that serves as an antigen. This component then stimulates the immune system within the fish against a particular pathogen.

TRADITIONAL VACCINES

Such vaccines typically come in one of two different types– inactivated vaccines and attenuated vaccines. Both involve giving the fish a dose of the pathogen we want to protect the fish against, but inactive vaccines use dead pathogens whilst attenuated vaccines use live pathogens in a weakened form so they don't actually cause illness.



Attenuated vaccines can provide better immunity than inactivated vaccines, and can also be used to treat young fish (by immersion).However since they use a live pathogen, there is a small risk that some residual virulence may remain and the treated animal – or those that come in contact with the animal, could become infected.Most successful use of inactivated vaccine has been against furunculosis (*Aeromonas salmonicida*) in salmon. Two live attenuated vaccines have been developed and are commercially available in the U.S. for enteric septicemia of catfish (caused by the bacterium *Edwardsiella ictaluri*) and columnaris disease (caused by the bacterium *Flavobacterium columnare*).

MOVING FORWARD

Thanks to advances in fields such as genetics, immunology, and biotechnology, new methods for fish vaccines have emerged in recent years that can be developed and produced over much faster time frames.

Replicating recombinant vector vaccines consist of a fully competent viral vector backbone engineered to express an antigen from a foreign transgene. Live vaccines replicate within the host. Most live virus vaccines in use today are attenuated, their reduced virulence typically achieved by adapting the wild-type virus to a new environment.

DNA vaccines against other types of fish pathogens have so far in limited use and vaccination strategy is more complicated. The

primary function of DNA vaccines is a bacterial plasmid DNA containing a construct for a given protective antigen, is to establish specific and long-lasting protective immunity against diseases where conventional vaccines fail to induce protection.Intramuscular injection of DNA vaccines has been successfully used against viruses such as infectious haematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV)diseases in fish.

Subunit vaccines are prepared from proteins or sugars derived from the disease-causing organism. Synthetic peptide vaccines are produced from short sequences of amino acids prepared synthetically to act as antigens.Against infectious pancreatic necrosis virus (*IPNV*) in farmed salmonid fish disease subunit vaccine are prepared fromVP2 and VP3 capsid proteins and used as oralvaccine inCanada, USA.

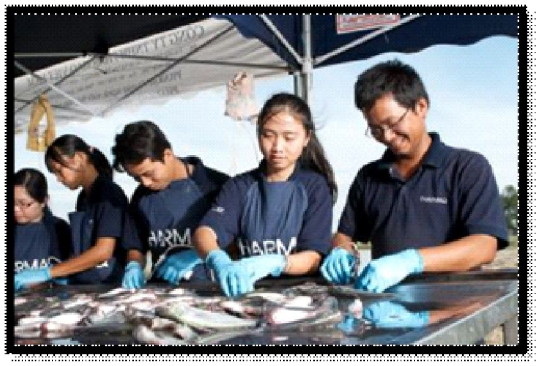
Different routes of delivery of vaccines in fish

Parameter	Oral delivery	Immersion delivery	Injection delivery
Efficacy	Low	Low/moderate	High
Labor input	Low labor input	Low labor input	Intensive labor input
Size of fish	Unlimited	Unlimited	Limited
Individual handling of fish	No	No	Yes
Open cages in seawater	Highly applicable	Not easily applicable	Not easily applicable
Closed systemvessels in freshwater	Highly applicable	Highly applicable	Applicable
Stress	Low	Low	High
Route of exposure	Gut/intestine	Natural portals of entry	Parenteral

The route of administration of vaccine is a vital factor influencing the efficacy and feasibility of vaccination. Injection, bath, and oral are the major administration routes used in aquaculture. Immersion vaccination is currently the most suitable method for mass vaccination of fish. As the fish is immersed in the vaccine solution, all mucosal surfaces including skin, gills, nostrils, eyes, vent and intestinal surfaces are exposed to antigens in the vaccine solution.Oral and bath immunizations (mucosal routes) are the ideal way for fish at all life stages, especially the larval stage when fish are often most susceptible to viral disease.But poor performance of oral vaccines is due to result of antigen degradation during passage through the hostile "stomach" like environment prior to reaching the second segment of the intestine where absorption takes place. It is to be noted that some *teleost fish have no anatomical stomach* at all & it produces both HCl and enzyme(s) from a single kind of cell. Injection is by far the most effective route, providing the highest protection.However, it is labour-intensive, causes handling stress to fish, and it is not feasible to vaccinate large numbers of juvenile fish via injection administration.Recently, automated vaccinating machines have been introduced that can lower labour costs and reduce stress to the fish.However, progress has been made regarding encapsulated technology of vaccine antigens which can be mixed with food for oral administration to increase it's stability.Nanotechnology has helped to formulate efficient vaccine delivery system that protects encapsulated antigen until it reaches to target area through gut & maintains sustained release.Other levels of commercial application

include intramuscular injection of DNA vaccines, nasal vaccination and hyperosmotic pretreatment before immersion vaccination.

ALPHA JECT® Panga 2 injection vaccine is given to *Pangasius* fish in Vietnam.The fish are netted out in small groups, drained for a few seconds from sea water and immersed in the dilution of vaccine.



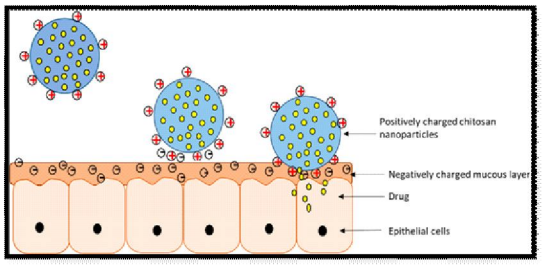
Why thinking of nanovaccines

When oral vaccination is most preferred but it suffers its degradation in the gastrointestinal tract.In contrast,encapsulating antigenic materials using several polymeric and lipid-based nanoparticle carriers could be an effective approach.Nanoparticle systems possess adjuvant properties that can enhance the efficacy of the antigens. An ideal vaccine carrier protects the structural integrity of the antigen and having effective delivery systemto reach mucosal surface in order to produce sufficient mucosal, humoral and cellular responses. Due to their very small size, nanoparticles enter living cells through cellular endocytosis.DNA vaccine incorporated into nanoparticles may be a more promising approach as the size of particle influences the delivery to a particular type of immune cells ensuring optimal antigen presentation, for example nanoparticles tends to be phagocytized preferentially by dendritic cells. This in turn may result in presentation via MHC class I and activation of the specific CTL(cytotoxic T lymphocyte)response typical of viral infections. Microparticles are more likely to be phagocytosed by macrophages and the antigens presented via MHC class II, generating a humoral response.

Nanovaccines

Nanotechnology is the study & application of extremely small things (about 1-100 nanometer or nm or 0.000000001 m) and this term is used across all other science fields. Nanoparticles are known to exhibit interesting properties different from their parent material. Due to their nano size, nanoparticles can be taken up by cellular endocytosis mechanism which facilitate the cellular uptake of antigens and increase the ability of antigen presentation.

Studies have demonstrated that application of nanotechnology increases solubility, stability, targeting, biocompatibility and permeability of vaccines. Nanovaccines, thus developed are made of nanoparticles formulated with antigens either encapsulated within or adsorbed on to the surface against which an immune response is desired. Numerous vaccine nanocarriers have been designed and investigated for their utility in the delivery of antigens and adjuvants to immune cells.The most explored nanoparticles in fish vaccine studies are synthetically derived polymeric PLGA and chitosan (deacetylation of chitin,which is the *structural* element in the exoskeleton of crustaceans like crabs, shrimp etc.) for administration of viral as well as bacterial antigens.Chitosan can be earmarked as a "green nanoparticle". It is highly abundant, biodegradable and biocompatible, making it an attractive candidate for vaccine delivery.Nanoconjugation of bicistronic DNA vaccine against *Edwardsiella tarda* using chitosan nanoparticles have shown protective efficacy and immune modulatory effects in *Labeo rohita* vaccinated by different delivery routes. Nanoparticles can boost the immune response in multiple ways. They can induce inflammatory reaction at the injection site, which recruits immune cells to the proximity of antigens, a phenomenon termed "reverse targeting".Phagocytic cells recruited to the injection site can phagocytize and subsequently digest the nanoparticles if they are biodegradable.



Schematicrepresentation of chitosan loaded nanoparticles (NP)structure and interaction with the mucus layer.NP upon reaching the mucosal layer bind to the negatively charged mucus by virtue of electrostatic attraction and release the drug over time.

Types of nanoparticles & their merits & demerits.

Type of nanoparticles	Merits	Demerits
Polymeric nanoparticles	Better immunogenicity can be obtained by easy modification of surface properties, biodegradable and targeted antigen delivery	Low aqueous solubility and synthesis require use of organic solvents, low antigen loading, premature release of antigens, insufficient antigen protection
Inorganic nanoparticles	Easy to modify, less chances of premature release and better protection of adsorbed antigens	Low aqueous solubility and low biodegradability
Nanoliposomes	Possess intrinsic adjuvant properties, accommodates both hydrophilic and lipophilic antigens and relatively stable in gastrointestinal fluids when modified	Low mucus penetration, limited antigen loading and poor gastrointestinal stability of naked liposomes.
ISCOMS	Easy to encapsulate and built in adjuvant property of Quil A	Do not form depot and difficult to incorporate hydrophilic antigens
Virus like particles	Possess self-adjuvant properties, mimics original virus and high gastrointestinal stability	Lack of reproducibility
Nanoemulsions	Possess self-adjuvant properties, encapsulates both hydrophilic and lipophilic antigens	Premature release of antigens and poor gastrointestinal stability

Hyaluronic acid (HA) is another natural polymer composed of D-glucuronic acid and N-Acetyl-

Dglucosamine and is a component of cartilaginous tissue.It is biocompatible, biodegradable, hydrophilic and due to high abundance in nature and makes it as one of the attractive candidate nanoparticles for vaccine delivery.Alginate is an extract of naturally available brown algae and also it can be found as a polysaccharide in some bacteria. It is also biodegradable, biocompatible, non-toxic, acid resistant, mucoadhesive and most suited for oral vaccine delivery.Further, the nanoparticles can be classified as biodegradable or non-biodegradable based on their properties to get decomposed in biological system. In general, the other forms of nanoparticles used in vaccine studies include virus-like particles (VPL's),nanoliposomes, immunostimulating complexes (ISCOMs), nanoemulsions and metal nanoparticles.

There are several inorganic nanoparticles based on carbon, calcium phosphate, gold, silver, silicate, aluminium, titanium etc., among which carbon nanotubes (CNTs) and calcium phosphate are evaluated as vaccine delivery systems in fish vaccines.CNT can be listed as emerging nanoparticles in the biomedical research and are investigated as antigen delivery systems. The inorganic nanoparticles have good adjuvant properties and stabilities but they have certain limitations in their chemistry and physical properties.

Plasmid (pGPD + IFN) **constructed ——— àchitosan NPs———-àincorporated into feed—-à DNA plasmid enters cells(myocytes, APC) ——à target cells uptake DNA by endocytosis —-à translocates to the nucleus —-à expression of the DNA-encoded protein antigen—à antigen is produced by the host cellular machinery—-à antigen protein is degraded and presented by major histocompatibility complex (MHC)-I in immune cells———-à directly stimulate naïve $CD8^+$ T cells———-àvaccinated groups evade the bacterial infection elucidating the protective efficacy

**Bicistronic DNA vaccine (designated as pGPD + IFN as adjuvant) containing a regular antigenic gene (glyceraldehyde-3-phosphate dehydrogenase gene of *Edwardsiella tarda*) along with an additional immune adjuvant gene (Interferon gamma gene of *Labeo rohita*)DNA vaccine can be delivered effectively by oral or immersion route.

Global fish vaccine manufacturers

Aquaculture vaccines is the fastest growing input in the global aquaculture market. The major commercial markets for these vaccine manufacturing companies are currently, the salmon and trout industries in Northern Europe, Chile, Canada and the USA. Till now, no known vaccines are marketed or used in India despite the fact that market attractiveness is very high[2].Recently Vietnam aquaculture industry got the government approval for use of ALPHA JECT® Panga 2 injection vaccine that provides protection against the main diseases in the *Pangasius* fish.

Commercial vaccines are available for the catfish industry in the USA and, on a smaller scale, for European seabream, seabass and tilapia. Some limited-use, locally developed

vaccines are also available in countries such as China, Russia, Spain and Germany. Key players in the market include: Pharmaq AS (Zoetis, LLC) (Norway), Merck Animal Health (Merck & Co., Inc.) (USA),KoVax Ltd. (Part of Phibro Animal Health) (Israel),Hipra (Spain),Tecnovax SA (Argentina),Veterquimica S.A. (Chile), Nisseiken Co. Ltd. (Japan),Virbac S.A. (France), Elanco (USA),Kyoritsuseiyaku Corporation (Japan)& few others.

MERCK Animal Health is reputed for marketing following vaccines-

NORVAX® Minova 6: Inactivated, multivalent vaccine against furunculosis, classical vibriosis, coldwater vibriosis, wound disease and infectious pancreatic necrosis (IPN) for intraperitoneal injection in Atlantic salmon.

AQUAVAC® Strep Sa 2: Vaccine for the active immunization of susceptible fish species to reduce mortality and disease due to Streptococcosis caused by *Streptococcus agalactiae*.

AQUAVAC® ERM (ORAL): Inactivated vaccine against Enteric Redmouth Disease caused by *Yersinia ruckeri* (Hagerman strain) in rainbow trout (*Oncorhynchus mykiss*). Available as an immersion vaccine as well as an oral administration vaccine.

AQUAVAC® VIBRIO: Inactivated vaccine against vibriosis caused by *Vibrio anguillarum* serotype OI and O2"" (*V. ordalii*) in rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*).

Conclusion

Nanotechnology-based gene delivery vectors have shown tremendous potential at overcoming physiological and biochemical barriers towards efficient gene delivery. While the nanoparticles have certain unique features & shown the undisputable potential for applications fish vaccine delivery, but the very nature of these particles have negative effects as well [3]. Nanoparticles can cross the blood brain barrier (BBB), the applications have to be made carefully as it may cause serious troubles. Current research focuses on elucidating the toxicity of nanoparticleswhich are postulated to range from inûammatorycell inûltration &cellular necrosis to ROS(reactive oxygen species)-induced apoptosis.More research is still needed regarding nanoparticles-based vaccine delivery platforms.

Advances in genome sequencing of pathogens have accelerated the opening of opportunities to investigate new approach of vaccine development. Recently, the genome of salmon and several other fish species have been fully sequenced. Development of polyvalent vaccines and standardization of a vaccination calendar with molecular biology and modern technologies can develop new dimension of vaccination. Plant-based edible fish vaccines can also contribute a lot in the field of fish vaccination.

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