Chapter 65

Characterizing Stability of Fish Vaccine Antigens Encapsulated in Plant-Based Nanoparticles

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ABSTRACT

Vaccination is essential for aquaculture in prophylaxis maintenance or preservation of fish health and productivity; however, the classical vaccine delivery systems have certain limitations. Here we review nanoparticle-based antigen delivery systems. The plant-derived nanoparticles are distinct as they have been shown to be bio-compatible, biodegradable and capable of targeted delivery making them attractive candidates. Bacterial, viral and parasitic antigens are regular targets used in fish vaccines; these who have their effective ability influenced by parameters such as pH of the environment, temperature and chemical oxidation. Some plant-based nanoparticle e.g. zein and chitosan types exhibit potential resistance to immune cell degradation which is beneficial for targeted delivery. Encapsulation efficiency can be improved by offering a wide range of antigen encapsulation techniques like emulsion, ionic gelation and coacervation. Advances in targeting for the delivery of Ag-loaded NPs require a comprehensive characterization which entails particle size, morphology as well as exclusion/conformation to some antigen loading and techniques such circular dichroism which show the conformational stability. Studies in vitro exhaustively analyze various facets such as the release kinetics, bioavailability and bioactivity besides assessing interactions with antigen-presenting cells while those in vivo examine immunogenicity, bio-distribution and protective activity using appropriate fish models to establish effectors having greatest therapeutic relevance. They can be turned into multivalent vaccines, formulated with adjuvants and targeted delivery. Further areas of research include using omics, modeling tools needs to be explored alongside stabilizing strategies and the concept around manufacturing (scale-up) and safety. Therefore, conferment of enhanced antigen stability and bioavailability as well as improved immunogenicity for fish vaccine antigens by encapsulation in plant-based nanoparticle formulations could promote effective vaccination strategies sustainable in aquaculture.

KEYWORDS
Aquaculture vaccination, Antigen delivery, Fish immunology, Plant-based nanoparticles, Vaccine formulation

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INTRODUCTION

Aquaculture, or the farming of aquatic organisms around the world has grown rapidly to meet global demand for seafood. This, however, may increase the chances of disease outbreaks on fish health and productivity. Furthermore, vaccination is key to reducing these risks and ensuring the sustainability of aquaculture. Vaccination is the best possible option for combating infectious diseases in aquaculture, thereby reducing antibiotic usage and also minimizing economic returns. Vaccines confer extended efficacy against key bacterial, viral and parasitic pathogens that increase the survival of fish throughout growth or production periods (Liu et al., 2024). Effective vaccination programs provide substantial savings by reducing the costs of disease outbreak which include mortality losses, growth retardation and treatment expenditures. This cost-efficiency is of particular importance in aquaculture where disease outbreaks can lead to significant economic losses for farmers (Mukaila et al., 2023). Fish vaccination is essential to avoid the need for antibiotics which nowadays has

become a serious problem in many parts of the world concerning resistance. The more that antibiotics are employed in aquaculture, the greater the probability for multiple drug-resistant bacteria to manifest and this is by no means innocuous as it pertains both to animal health but human wellbeing too. In turn, this could likely to lower the requirements for antibiotic treatments by way of disease outbreaks and make a significant contribution towards sustainability in the aquaculture practices (Imtiaz et al., 2023).

Vaccination also benefits fish welfare and product quality and the vaccinated fish usually grow more, consume their food better and present themselves with higher product quality than unvaccinated siblings. This is not only of benefit for the farmers but also adds to a better image in general and acceptance of aquaculture products by consumers (Miccoli et al., 2021). Fish vaccination has proven advantageous but the creation of safe and affordable vaccines for different aquaculture species is still facing a number of difficulties. Both of these barriers can be overcome via ongoing research and creativity, which contribute to the overall sustainability as well as financial success within aquaculture (Priya and Kappalli 2022). Vaccination of fish is an important tool to avoid infections in aquaculture and has the potential to increase animal health, welfare, product quality minimize use of antibiotics. The future sustainability and resilience of aquaculture operations depend on implementing effective vaccination strategies as the industry continues to grow (Flores-Kossack et al., 2020).

Conventional ways of vaccine delivery in aquaculture like injection and immersion confront several barriers. The problem with injection vaccination is that it works, but also laborious (handles the fish several times), causes stress to the fish and high incidence of adverse reactions or handling-related injuries. Injection vaccination have the tendency to cause side effects like adhesion, pigmentation and tumor near where vaccinations are injected thereby leads death sometimes. Immersion vaccination, in contrast, rarely achieves commercial success due to the low efficacy of antigen intake and limited immune response. It can be more efficient but still dependent on the type of antigen, to immersion time not say about water quality and therefore protection levels also vary enormously (Du et al., 2022).

As a potential alternative to the conventional vaccination approaches, nanoparticle-mediated antigen delivery systems have recently been researched. These nanoparticles can shield antigens from degradation, enhance their bioavailability and selectively deliver them to immune cells providing an edge for the development of more efficient vaccines (Bezbaruah et al., 2022). Nanoparticle drug delivery system has certain advantages, such as better antigen stability and release of mucosal adjuvant that could reduce the injection burden of vaccination remarkably (Elumalai et al., 2024). The plant based nanoparticles have been the most attractive among various nanoparticle platforms as vaccine carriers in aquaculture. Recently, plant-based nanoparticles received increasingly more attention in the drug delivery research community due to their biodegradability and potential lower immunogenicity with the ability of synthetic polymer degradation products might induce innate immunity (Mondal and Thomas 2022).

Typically, one of the most common plant based nanoparticles include zein (corn), gliadin (wheat) and chitosan which are derived from shellfish. It is possible to use these nanoparticles as carriers, either by forming a protective shell around an antigen-loaded core or surfacing the nanoparticle with antigens (in this case without protecting them from myriad proteases in solution) thus targeting particles for uptake by immune cells (Nguyen et al., 2022). Plant-based nanoparticles, especially those with proteinaceous nature have been evaluated as potential vaccine carriers for fish (Table 2). Zein nanoparticles loaded with a viral antigen were able to serve as an entry for inducing active immunity against nervous necrosis virus in European sea bass (Angulo et al., 2022). Additionally, improved immunity response and increased survival against Streptococcus iniae disease was also observed upon incorporating chitosan nanoparticles encapsulating a bacterial antigen in Nile tilapia (Suwanbumrung et al., 2023). The attractive advantages of the plant-based nanoparticles are evident, but several daunting challenges need to be overcome before exploring these tiny versatile platforms further and employ more advanced polyanhydrides such as copolymers in MN design including fine tuning of antigen loading/releasing kinetics, long-term stability issues like solvent compatibility during synthesis/storage and scaling up for high volume manufacturing (Basu et al., 2021).

Fish Vaccine Antigens

In fact, vaccine antigens are the key components that induce protective immune responses against particular pathogens. In the area of fish vaccination, a great number of antigens from bacteria/pathogens and also parasites have been attempted for use in vaccine formulae (Schijns et al., 2021).

Common Bacterial, Viral and Parasitic Antigens used in Fish Vaccines

Bacterial Antigens

Gram-negative bacterial antigens have probably been the most extensively studied and applied in fish vaccines for the control of diseases caused by these microorganisms. The bacterial pathogens which are targeted by phage biocontrol products include some of the Vibrio species (V. anguillarum, V. salmonicida), Aeromonasspp (A. salmonicida, A. hydrophila) and Yersinia ruckeri responsible for substantial economic losses incurred in aquaculture industry [6]. The latter may be derived from outer membrane proteins, lipolysacharides or extracellular products and have been used successfully in fish vaccines (Singh et al., 2023).

Viral Antigens

Viral infection can be one of the most threatening and as a result, vaccination has mainly targeted viral pathogens causing diseases such as infectious pancreatic necrosis virus (IPNV), Infectious hematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV) in salmonids or betanoda virus for marine species. Glycoproteins and capsid proteins present on the surface of virus whether in recombinant subunit vaccines or by using whole-virus preparation (such as inactivated) have been evaluated extensively (Mugimba et al., 2021).

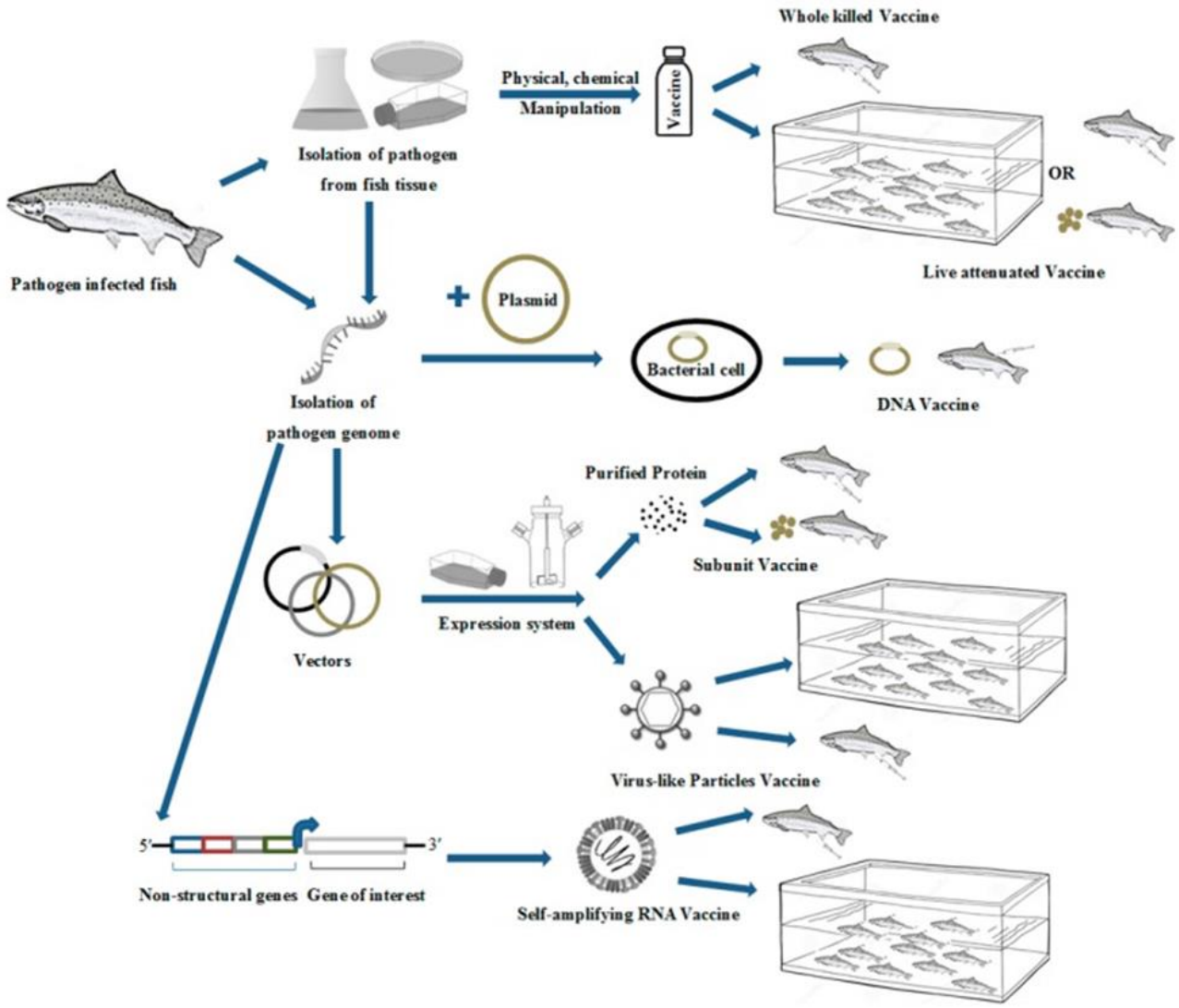

Fig. 1: Various approaches for fish vaccine development (Ma et al., 2019)

Parasitic Antigens

Parasitic diseases also pose a challenge for aquaculture and vaccine development has included protozoan and metazoan parasites. Antigens from Ichthyophthirius multifiliis, a ciliated protozoan have been investigated, using vaccination research for white spot disease in freshwater fish as an example. Antigens of the monogenean parasite Gyrodactylus salaris to be used for prophylaxis against salmonid gyrodactylosis have also been examined (see below) (Buchmann 2022).

Structural and Functional Properties of Antigens

Antigens are usually proteins or polysaccharides which can be recognized by the immune system and that induces either a specific antibody response, sometimes referred to as humoral immunity. Antigenic properties are essential in determining the structural and functional characteristics of antigens, hence antigen design is necessary for effective vaccine development (Kapingidza et al., 2020).

Protein Antigens

Proteins are antigens belonging to almost all bacterial, viral and parasitic strains; they perform various structural forms and have specific functional regions. These features may alter the level of immunogenicity, stability and mode-of-action. Examples of this include the selection and testing of surface-exposed molecules or those involved in

pathogenesis/virulence as vaccine antigens, because it has been hypothesized that host immunity against these bacterial proteins may prevent invasive infections caused by bacteria expressing them (Mishra et al., 2020).

Antigen-Polysaccharide

Lipo-polysaccharides (LPS) from gram−bacteria capsular polysaccharides from some pathogens carbandoglycans are able to induce the production of antibodies against protein antigens, without being as immunogenic as other proteins in fish (Gao et al., 2023).

Table 1: Commercial vaccines used in fish against various pathogens

Vaccine name	Administration	Pathogen	Target Fish Species
Bacterial pathogens			
ALPHA JECT® micro 4	injection	*Aeromonas salmonicida, Vibrio anguillarum, Vibrio salmonicida*	Atlantic salmon (*Salmosalar*)
Alpha ERM Salar	injection	*Yersinia ruckeri*	Atlantic salmon (*Salmosalar*)
fiALPHA JECT LiVac® SRS	injection	*Piscirickettsia salmonis*	Atlantic salmon (*Salmosalar*), Rainbow trout (*Oncorhynchus mykiss*) Coho salmon (*Oncorhynchus kisutch*)
ALPHA JECT® micro 1 Tila	injection	*Streptococcus agalactiae*	Nile tilapia (*Oreochromis niloticus*)
ALPHA JECT® Panga 2	injection	*Aeromonas hydrophila, Edwardsiella ictaluri*	Iridescent shark (*Pangasianodon hypophthalmus)*
ALPHA JECT® 5–3	injection	*Aeromonas salmonicida, Vibrio salmonicida, Vibrio anguillarum, Moritellaviscosa*	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 3000	injection	*Aeromonsa salmonicida, Vibrio anguillarum*	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 2000	injection	*Vibrio anguillarum, Photobacterium damsela*	Sea bass (*Dicentrarchus labrax*)
Aeromonasveronii vaccine	injection	*Aeromonas veronii*	Sea bass (*Dicentrarchus labrax*)
ALPHA DIP® Vib	immersion	*Vibrio anguillarum*	Sea bass (*Dicentrarchus labrax*)
Alpha ERM Salar	immersion	*Yersinia ruckeri*	Atlantic salmon (*Salmosalar*)
ALPHA DIP® Vibrio	immersion	*Vibrio anguillarum*	Sea bass (*Dicentrarchus labrax*)
ALPHA DIP® 2000	immersion	*Vibrio anguillarum, Photobacteriumdamsela*	Sea bass (*Dicentrarchus labrax*)
AQUAVAC® Strep SA	injection	*Streptococcus agalactiae*	Nile tilapia (*Oreochromis niloticus*)
AQUAVAC® Strep Sa1	injection	*Streptococcus agalactiae*	Nile tilapia (*Oreochromis niloticus*)
AQUAVAC® Strep Si	immersion /injection	*Streptococcus iniae*	Nile tilapia (*Oreochromis niloticus*), Sea bass (*Dicentrarchus labrax*)
AQUAVAC® RELERA™	immersion	*Yersinia ruckeri*	Rainbow trout (*Oncorhynchus mykiss*)
AQUAVAC® VIBRIO	injection/ immersion	*Vibrio anguillarum*	Rainbow trout (*Oncorhynchus mykiss*)
AQUAVAC® Vibrio Pasteurella	injection	*Photobacterium damsela*	European sea bass (*Dicentrarchus labrax*)
AQUAVAC® ERM	immersion/oral	*Yersinia ruckeri*	Rainbow trout (*Oncorhynchus mykiss*)
BLUEGUARD® SRS ORAL	oral	*Piscirickettsia salmonis*	Atlantic salmon (*Salmosalar*), Rainbow trout (*Oncorhynchusmykiss*), Coho salmon (*Oncorhynchuskisutch*), *Chinook salmon* (*Oncorhynchus tshawytscha*)
BLUEGUARD® SRS INYECTABLE	injection	*Piscirickettsia salmonis*	Atlantic salmon (*Salmosalar*), Rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*)
Viral pathogens			
ALPHA JECT micro® 1 PD	injection	Salmonid alphavirus 3	Atlantic salmon (*Salmosalar*)
ALPHA JECT® micro 1 ISA	injection	Infectious salmon anaemia virus	Atlantic salmon (*Salmosalar*)

ALPHA JECT® 1000	injection	Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*), Rainbow trout (*Oncorhynchus mykiss*)
ALPHA JECT micro® 1 Noda	injection	Nervous necrosis virus	Sea bass (*Dicentrarchus labrax*)
AQUAVAC® IridoV	injection	Iridovirus	Asian sea bass (*Lates calcarifer*), Nile tilapia (*Oreochromis niloticus*)
BLUEGUARD® IPN ORAL	oral	Infectious pancreatic necrosis virus	Atlantic salmon *(Salmosalar*), *Rainbow trout* (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*)
BLUEGUARD® IPN INYECTABLE	injection	Infectious pancreatic necrosis virus	Atlantic salmon *(Salmosalar*), Rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*)
Combined vaccines against bacterial and viral pathogens			
ALPHA JECT® IPNV-Flavo 0,025	injection	*Flavobacterium psychrophilum*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT micro® 7 ILA	injection	*Aeromonas salmonicida, Vibrio anguillarum, Vibrio salmonicida, Moritella viscosa*, Infectious salmon anaemia virus, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT micro® 6	injection	*Aeromonas salmonicida, Vibrio anguillarum, Vibrio salmonicida, Moritellaviscosa*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® micro 3	injection	*Vibrio ordalii*, *Piscirickettsia salmonis*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® micro 2	injection	*Piscirickettsia salmonis*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*), Coho salmon (*Oncorhynchus kisutch*), Rainbow trout (*Oncorhynchus mykiss*)
ALPHA JECT® 6–2	injection	*Aeromonas salmonicida, Vibrio salmonicida, Vibrio anguillarum, Moritellaviscosa*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 5–1	injection	*Piscirickettsia salmonis, Vibrio ordalii*, *Aeromonas salmonicida*, Infectious pancreatic necrosis virus, Infectious salmon anaemia virus,	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 4–1	injection	*Piscirickettsia salmonis, Vibrio ordalii*, *Aeromonas salmonicida*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 2–2	injection	*Aeromonas salmonicida*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
BLUEGUARD® SRS + IPN INYECTABLE	injection	*Piscirickettsia salmonis*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*), Rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*)
BLUEGUARD IPN + SRS+ As+Vo + ISA INYECTABLE	injection	*Piscirickettsia salmonis, Vibrio ordalii, Aeromonas salmonicida*, Infectious pancreatic necrosis virus, Infectious salmon anaemia	Atlantic salmon (*Salmosalar*)
Viral pathogens			
ALPHA JECT micro® 1 PD	injection	Salmonid alphavirus 3	Atlantic salmon (*Salmosalar*)
ALPHA JECT® micro 1 ISA	injection	Infectious salmon anaemia virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 1000	injection	Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*), Rainbow trout (*Oncorhynchusmykiss*)

ALPHA JECT micro® 1 Noda injection	Nervous necrosis virus	Sea bass (*Dicentrarchuslabrax*)
AQUAVAC® IridoV injection	Iridovirus	Asian sea bass (*Lates calcarifer*), Nile tilapia (*Oreochromis niloticus*)
BLUEGUARD® IPN oral ORAL	Infectious pancreatic necrosis virus	Atlantic salmon *(Salmosalar*), *Rainbow trout* (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*), Chinook salmon (*Oncorhynchus tshawytscha*)
BLUEGUARD® IPN injection INYECTABLE	Infectious pancreatic necrosis virus	Atlantic salmon *(Salmosalar*), Rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*Oncorhynchus kisutch*),
Combined vaccines against bacterial and viral pathogens		
ALPHA JECT® injection IPNV-Flavo 0,025	*Flavobacterium psychrophilum*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT injection micro® 7 ILA	*Aeromonas salmonicida, Vibrio anguillarum, Vibrio salmonicida, Moritellaviscosa*, Infectious salmon anaemia virus, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT injection micro® 6	*Aeromonas salmonicida, Vibrio anguillarum, Vibrio salmonicida, Moritellaviscosa*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® injection micro 3	*Vibrio ordalii*, *Piscirickettsia salmonis*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® injection micro 2	*Piscirickettsia salmonis*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*), Coho salmon (*Oncorhynchus kisutch*), Rainbow trout (*Oncorhynchus mykiss*)
ALPHA JECT® 6–2 injection	*Aeromonas salmonicida, Vibrio salmonicida, Vibrio anguillarum, Moritellaviscosa*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 5–1 injection	*Piscirickettsia salmonis, Vibrio ordalii*, *Aeromonas salmonicida*, Infectious pancreatic necrosis virus, Infectious salmon anaemia virus,	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 4–1 injection	*Piscirickettsia salmonis, Vibrio ordalii*, *Aeromonas salmonicida*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)
ALPHA JECT® 2–2 injection	*Aeromonas salmonicida*, Infectious pancreatic necrosis virus	Atlantic salmon (*Salmosalar*)

Factors Affecting Antigen Stability

Antigen stability is vital to vaccine potency and effectiveness. Antigen stability can be impacted by a variety of factors, including:

pH

Antigens, especially proteins can degrade their structure and functionality due to a simple pH modification. Denaturation, aggregation or degradation of antigens from pH conditions that are either too high or too low and will reduces the immunogenicity. One strategy in vaccine formulation and storage is to maintain the associated antigen stability within a suitable pH range (Delfi et al., 2021).

Temperature

You can make antigen stability a high-risk item here as temperature changes will considerably affect the will of a nucleotides. Proteins unfold and/or aggregate and may lose biological activity at high temperatures, but freeze-thawing can promote structural changes that cause denaturation (Ma et al., 2020).

Oxidation

Oxidative stress can alter the protein structure and potentially inactive antigens, namely those with cysteine residues or other amino acids that are easily oxidized. Proper antioxidants or packaging materials can thus help in controlling oxidation and maintaining antigen quality (VasileandBaican 2021).

Proteolytic Degradation

Protein-based antigens are often unstable and become degraded by proteases where these enzymes may be present in the formulation or secreted to process endocytosed antigen. Protease inhibitors or stabilizing agents will likely be required to protect against premature degradation (Pishesha et al., 2022).

Adjuvants and Excipients

Vaccine formulations also differ in the adjuvant (immunologic stimuli) and excipient (inactive ingredient that stabilizes the antigen) (Qi and Fox, 2021). Some adjuvants or excipients can impact antigens in a way that changes their structure or potency. It is essential to know the antigens stability which effect its effective and stabile fish vaccines. It is lethal to the virus, however it also keeps antigen conformation and retains optimal vaccine function with suitable formulation strategies such as stabilizers, lyophilization or encapsulation techniques (Du et al., 2022). Addressing the issues of antigen stability, and including relevant bacterial, viral or parasitic antigens in vaccines may allow researchers to deliver effective fish vaccines that ensure long-term success for a sustainable aquaculture industry (Sahoo et al., 2020).

Plant-Based Nanoparticles for Antigen Encapsulation

Over the few last years, plant-based nanoparticles (NPs) have appeared to be an innovative antigen delivery system in vaccine formulation especially for aquaculture species. The nanoparticles described in this work are bio-sourced from different types of plant-based biomaterials and present several benefits over the synthetic ones (Stander et al., 2022).

Types of Plant-based Nanoparticles

Protein-based Nanoparticles

Plant proteins, including zein (corn origin), gliadin wheat or soy protein are widely investigated for the synthesis of nanoparticles. Zein nanoparticles have been recently investigated in detail for their considerable biocompatibility, degradability and also controlled antigen encapsulation/release capabilities (Martínez-López et al., 2020).

Lipid-based Nanoparticles

Plant derived lipids like those found in oils and waxes can be formed into nanoparticles, called nanostructured lipid carriers (NLCs) or solid lipid nanoparticles (SLNs). Efforts at exploring the encapsulation and delivery of antigens within vaccine formulations by lipid-based nanoparticles (Xu et al., 2022).

Advantages and Limitations of Plant-based Nanoparticles

Advantages

Biocompatibility and biodegradability: Plant-based nanoparticles are capable to reduce the risk of adverse reactions and facilitate their clearance from organism due to biocompatibility/biodegradability (Kučuk et al., 2023). Nanoparticles are used as delivery systems for antigens, and they provide protection against antigen degradation while allowing controllable release to increase bioavailability of the antigen in immune cells (Luzuriaga et al., 2021). Adjuvant properties as a side note, some plant nanoparticles like chitosan has an inherent immune stimulating property which can acts as adjuvants to potentiate the desired response (Nordin et al., 2023). Plant-derived nano-technology may facilitate the mucousal or oral administration of vaccines, which can be useful for aquaculture (Ortega-Berlanga and Pniewski 2022).

Limitations

- Batch-to-batch variability: Plant-derived materials may inherently be variegated in composition and performance, which could influence the reproducibility of various nanoparticles formulations (Majeed et al., 2024).
- Scalability and manufacturing challenges: Scaling up plant-based nanoparticle production while maintaining quality and reproducibility is a battle in itself (Zhu et al., 2021).
- Potential immunogenicity: How bad is the potential immunogenicity of plant-derived materials which can be hardly useful for some applications (Umeoguaju et al., 2021).
- Regulatory implications: Although the use of plant-based nanoparticles in vaccine formulations is feasible, this approach may necessitate additional regulatory approvals and/or safety testing (Venkataraman et al., 2021).

Antigen Encapsulation Techniques and Efficiency

Several techniques have been explored for encapsulating antigens within plant-based nanoparticles including:

Emulsion-based techniques: the antigen is dissolved or dispersed within an organic solvent and mixed in emulsification with a water phase containing plant material. The subsequent elimination of the organic solvent produces nanoparticles loaded with antigen (Jamir et al., 2024).

Ionic gelation Antigens may also be encapsulated into nanoparticles via ionic interactions between oppositely charged polymers e.g. chitosan with tripolyphosphate (TPP). This ionic gelation leads to formation of nanoparticles with antigen entrapment (Di Santo et al., 2021).

Coacervation: a technique for physically separating an aqueous liquid into two immiscible phases in which one is enriched with plant based materials and the other contains antigen. The solvent can then be removed, leading to the nanoparticles with entrapped antigens (Yusree et al., 2021). The efficiency of antigen encapsulation relies on different factors such as nature of the antigen and plant matrix, method used for encapsulating and processing parameters. Since encapsulation efficiencies are governed by the same parameters it is of utmost importance that these have to be optimized such that not only can antigens withstand process stresses but also remain thermostable and bioreactive after being loaded (Klojdová et al., 2023). Therefore, through integrating the potential benefits of plant-based nanoparticles with specific encapsulation technologies, fish vaccine antigen delivery systems would reach higher efficacy levels and will drive both researches and manufacturers from academia into small companies. Nevertheless, mitigation of the shortcomings and legal aspects is important in order to ensure such nanoparticle-based vaccines can effectively be translated into realistic applications within aquaculture (Ahmed et al., 2023).

Characterization of Antigen-Loaded Nanoparticles

It is important that the loading antigen and chemistry of the nanoparticulate material being tested are accurately characterized as this information can impact their fluency, stability and immunological potential. Various methods are used to characterize these nanoparticles that include (Dong et al., 2021).

Particle Size, Polydispersity and Zeta Potential

The most valuable parameter is the particle size and its distribution which significantly affects bioavailability, biodistribution as well as cellular uptake of nanoparticles. Based on the size and PDI, DLS is a common technique used to measure hydrodynamic sizes of nanoparticles in suspension (Wang et al., 2020). Zeta potential, which provides information about the surface charge of nanoparticles is also an important parameter that influences their stability and interactions with biological systems as well as ability for antigen encapsulation and release. Electrophoretic light scattering or laser doppler electrophoresis is the method of choice for zeta potential measurements (Rasmussen et al., 2020).

Morphological Analysis (SEM, TEM, AFM)

Morphological analysis is important to recognize the shape, surface topography and internal structure of NPs which may results in different biological activity as antigen carrier. The surface morphology and topography of nanoparticles were analyzed using scanning electron microscopy (SEM) which gives high-resolution images (Zhang et al., 2021). Transmission electron microscopy (TEM) can render nanoparticle size, shape and internal structure at higher magnifications to identify probable defects or irregularities (Mast et al., 2020). Three-dimensional topographic images of nanoparticles are obtained by atomic force microscopy (AFM) at high resolution, which provides the analysis on surface features and roughness (Lutter et al., 2020).

Antigen Loading Capacity and Encapsulation Efficiency

Antigen loading capacity and encapsulation efficiency are the two essential parameters related to how well nanoparticle vaccine delivery systems perform. Antigen loading capacity: It is the measure of how much antigen was loaded or associated with the NPs, usually it's expressed as weight ratio of Ag/NP (Hong et al., 2020). It is the proportion of content in percentage that was encapsulated or associated with the nanoparticles compared to original antigen amount (Ryu et al., 2021). Antigen loading and encapsulation efficiency are quantified using a number of techniques, by:

Quantification by direct methods: the amount of antigen present in nanoparticle formulation can be quantified using techniques such as UV-Visible spectroscopy, Fluorescence spectroscopy or ELISA (Tabatabaei et al., 2021).

Methods for Indirect quantification: includes the separation of non-encapsulated or free antigen from nanoparticle suspension and then, evaluation of this separated part using conventional techniques such as centrifugation, ultrafiltration and size exclusion chromatography (Giordani et al., 2023).

These studies reveal the importance of determining the particle size, surface charge value and morphological characteristics as well as antigen loading/encapsulation efficiency to assess whether there are properties that can be associated with enhanced performance of vaccine delivery using these antigens loaded nanoparticles. These analyses give particularly useful insight into designing nanoparticle formulations, maintaining batch-to-batch consistency and predicting their fate in biological systems (Alqahtani et al., 2020). Additionally to meet regulatory compliance and support the quality and reproducibility of nanoparticle-based vaccine products through comprehensive characterization. Using the correct techniques of characterization and creating defined physicochemical parameters, researchers can help to enable the development and translation of these novel approaches for vaccine delivery systems in aquaculture (and other applications) by industrial producers (Ramos et al., 2022).

Stability Studies of Encapsulated Antigens

The antigen encapsulation stability of these types of delivery vehicles is an important parameter that ensures the

potency and efficacy of nanoparticle-based vaccine formulations. Stability studies: encapsulated antigens shall be evaluated for thermal, pH as well storage and conformational stability under various conditions (Diaz-Arévalo and Zeng 2020).

Thermal Stability (DSC, TGA)

The integrity and bioactivity of the encapsulated antigens are affected by thermal stability during storage, transportation or administration. Thermal properties of antigen loaded nanoparticles were investigated using thermal analysis techniques including: differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) (Cao et al., 2024). DSC thermal stability and compatibility of the encapsulated antigen with nanoparticle matrix are obtained from heat flow measurements associated to such transition temperature, whether it refers a melting point, glass transition or protein denaturation (Kaur et al., 2021), measures the sample mass loss vs temperature allowing you to provide thermal degradation temperatures or identify any potential dehydration, and/or possible decomposition (Wang et al., 2021).

pH Stability

Antigens, such as proteins are very sensitive to pH changes inducing structural rearrangement or denaturation or aggregation. The pH stability studies consist of incubating antigen-loaded nanoparticles in buffers at selected values and measuring the retained amount prior/after time points (Zhang et al., 2021One would then quantify how much of the intact antigen remains with different pH treatment using techniques such as size-exclusion chromatography, SDS-PAGE or enzyme-linked immunosorbent assay (ELISA) (Lieu et al., 2021).

Storage Stability (accelerated and real-time)

Storage stability studies are required for the calculation of shelf-life and long-term activity retention in case of antigen-loaded nanoparticles. These studies could be real-time or but not limited to accelerated. When assessing real-time stability, nanoparticle formulations should be stored under desirable conditions (e.g. refrigerated and room temperature) with an antigen release profile monitored at predetermined intervals for a extended period. Accelerated stability studies: These are the studies that include high temperatures and humidity conditions in order to fasten probable degradation processes. Estimation of shelf life: The data may be subjected to kinetic modeling and extrapolation methods (Reinhart et al., 2023). Size exclusion chromatography, ELISA or bioassays can be employed to follow the integrity and stability of antigen during storage studies across time (Le Basle et al., 2020).

Conformational Stability (CD, FTIR)

The preservation of antigenic, especially protein antigens in their native conformation is indispensable for any further development and utilization. Circular dichroism (CD) and Fourier-transform infrared spectroscopy (FTIR) are used, among others that help to elucidate the conformational stability of the encapsulated antigens (Parlar et al., 2021). CD spectroscopy can differentiate in the absorbance of left and right circularly polarized light inducing by optically active molecules.it gives an information about secondary andtertiary structure of proteins (Subadini et al., 2022). FTIR spectroscopy can be used to detect the vibrational modes of chemical bonds of a peptide bond, make it possible for different secondary structures such as α-helix and β-sheet specific identification in proteins modulate their conformational shifts (Yang et al., 2022). A deeper insight is gained into the thermal, pH and storage stability of antigens in NP formulations with the help of different analytical methods used during NPs-antigens incubation under varied conditions. Such studies are critical to optimize formulation parameters, develop appropriate storage conditions as well as maximize the long-term stability and potency of vaccine products based on nanoparticle (Shi and McHugh 2023)In addition, stability data are an essential prerequisite for regulatory approval and market release of these vaccine preparations. To provide a comprehensive demonstration of stable encapsulation and the potential for freeze drying, we believe that demonstrating these properties in closed studies will help increase the chances to translate innovative delivery system vaccines into practical use both at field (aquaculture or other) levels (Ingle and Fang 2023).

Factors Influencing Antigen Stability

Several factors, as the type of nanoparticles and their properties composition in plant-based nanoparticles, have impact on it; even consumption method how antigen is packedratio or modifying pharmacological compounds are related to (Kyriakoudi et al., 2021). Nanoparticles composition and surface properties: The type of protein, polysaccharide or lipid used to produce plant-based nanoparticles can have a major influence on the stability of encapsulated antigens. For example, zein nanoparticles have shown higher protective effect to antigens compared with protein based because of their hydrophobic nature and high ordered structure (Paliya et al., 2023).

Encapsulation Method and Conditions

Depending on both the specific method used to encapsulate antigens in plant-derived nanoparticles and conditions during the process of encapsulation, antigen stability can be dramatically affected. The use of organic solvents or harsh processing conditions (e.g. high temperatures, shear forces) for emulsion-based encapsulation techniques may negatively affect antigen stability, hence resulting in the loss/modifications/enhancement/neutralization of some epitopes on the

encapsulated antigens during formulation development. In contrast, gentle encapsulation methods such as ionic gelation or coacervation may protect antigen stability more effectively by excluding the material from denaturing conditions (Ramos et al., 2022). The way an antigen interacts with the nanoparticle matrix can greatly affect its stability. The presence of antigen surrounding particles has been suggested to stabilize the biologics by stronger electrostatic, or hydrophobic interactions between the antigen and nanoparticle components providing a protective environment so that protein cannot de-nature/denigrate. Conversely, weak or unfavorable interactions might result in antigen release or denaturation before the particles have been internalized. Such interactions are important for rational vaccine design and thus an understanding is required to formulate nanoparticles that can retain the antigenicity suitable for vaccines (Gomes et al., 2022).

In Vitro and *In Vivo* Evaluations

More detailed assessment by in vitro and in vivo studies of the performance and efficacy of a nanoparticle-based fish vaccine formulation is needed as well. These evaluations generally deal with effects of the carrier on release kinetics and bioavailability of encapsulated antigens, their preservation or modulation by respect to free forms, as well as uptake and processing by antigen-presenting cells (Attaya et al., 2021). The in vitro release studies are carried out to assess the kinetics of antigen release from nanoparticles prepared using plant repository and also for comparing it with animal origin materials. These studies give us an idea of the release profiles that may affect bioavailability and, thus, adjuvanticity or dangers posed by encapsulated antigens. Release studies are typically carried out by dialysis or diffusion based methods in which the nanoparticle formulation is loaded into a dialysis membrane/insert and released antigen is measured over time (Gómez-Mascaraque et al., 2021). The pH value, temperature or presence of enzymes and biological fluids were not always the same in every study for a given physiological environment (Yadav et al., 2023). Preserving the bioactivity and immunogenicity of antigens that are encapsulated with carriers is critical to induce effective immune responses in fish. For assessment of the biological activity such as potential antigens dance released to antigen–antibody fit or effect on immune cells, in vitro assays are performed. Miscellaneous in vivo immunogenicity studies such as adaptive immunity, cellular immune response or to determine whether the nanoparticle-encapsulated antigens can elicit a protective responses against pathogens infecting fish models are also documented (Zhang et al., 2021). Indeed, studies previously conducted with this vaccine format usually included immunizations that followed by monitoring the development of antigen-specific antibodies and cytokines or challenge exams right after stimulating the fish together with live pathogens to verify its protective role. Antigen presenting cells (APCs), such as dendritic cell and macrophages are crucial in the initiation of adaptive immune responses, having an impact on both antigen uptake ability and processing efficiency. Nanoparticle-encapsulated antigens are used to study uptake and intracellular trafficking in vitro with fish APCs or cell lines (Wang et al., 2021).

Flow cytometry, confocal microscopy and immunofluorescence assays techniques could be used to visualize or quantify the uptake of nanoparticles (with a fluorophore) or antigens by professional APCs. Second, APC activation and co-stimulatory molecules or cytokines are examined following administration of nanoparticle-encapsulated antigens. In vivo studies in fish models can additionally help to identify the bio-distribution and uptake of nanoparticle-encapsulated antigens by APCs from different lymphoid organs or tissues, pointing out the potential induction of a systemic or mucosal inflammatory profile (Zhao et al., 2024). Such in vitro and in vivo studies provide detailed information on the performance of different parameters i.e., release kinetics, bioavailability, bioactivity immunogenicity as well as possible fish immune system-interactions associated with nanoparticle-based vaccine formulations for application to protect against early marine pathogen infections. This is essential for the formulation optimization, assessing their efficacy and guaranteeing a high verticalization of this innovative vaccine delivery systems in aquaculture.

Challenges and Future Prospects

Plant-based nanoparticles have shown, unique and promising attributes for effective fish vaccine antigen delivery; nonetheless several inherent challenges need to be tackled along with exploring potential research directions in future that may steer their successful translation and practical implementation. The major bottleneck in the development of plant-based nanoparticle-like vaccines is scale-up and manufacturing. The move from small-scale laboratory fabrication to large volume commercial manufacturing may be complex, demanding a degree of product consistency and batch-to-batch uniformity. Furthermore, the bio-based nature of plant derived materials can result in compositional and property variance necessitating that future material are subject to strict quality control and standardized protocols. Solving these issues through the implementation of strong and scalable manufacturing platforms is an essential requirement for a successful commercialization process around those vaccine products.

The field of regulation for nano-particulate-based vaccines is ever-growing and requires guidelines or framework to affirm their safety as well as the efficacy. These concerns are also why a perfusion-based safety evaluation is needed for nanoparticle toxicity, because the bio-distribution and environmental issues related with nanoparticles. In addition, the regulatory authorities may demand further data and evidence to confirm that plant-derived nanoparticles are suitable for vaccine carriers especially on their immunogenicity, long stability and compatibility with the current process of producing vaccines. One of the attractive aspects about plant-based nanoparticles is their capability to deliver multivalent or combined vaccines. Synthetic vaccines using these nanoparticles may be engineered to include or surface-display multiple antigens, creating the ability to vaccinate against more than one pathogen at a time. Moreover, plant-based nanoparticles

could be combined with other vaccine entities such as immuno-stimulants or adjuvants to potentiate the immune response and offer multi strain protection. These combination strategies can offer insights into designing more effective and holistic vaccine formulations for aquaculture utilization. Current studies are investigating new plant-derived substrates and also different types of nanoparticle formulations for antigen delivery. It also explores the potential of using plant-based polysaccharides, lipids and biopolymers as a viable matrix for nanoparticles. Researchers are also investigating the evolution of targeted nano delivery systems, attaching ligands or antibodies onto nanoparticles to come into contact with immune cells for mucosal access.

Intercalation of advanced characterization methods, such as omics-based profiling and in silico modeling will enable a better comprehension to the interactions among nanoparticles-antigens-immune system leading toward rationalized design pathways for highly effective vaccine formulation. Future research will additionally focus on challenges associated with the stability, time-released manner and bioavailability of encapsulated antigens using new strategies to encase these vaccines and surface modifications in addition to including stabilizers or adjuvants. With the resolution of these challenges and an increasing trend in new research directions, it will be possible to maximize on the potential delivery systems for fish vaccine antigens based on plant-based nanoparticles leading to safe, effective and sustainable vaccination strategies predominantly intended towards maintaining healthy aquatic environments.

Summary of Key Findings

Proteins, polysaccharides and lipids are the key plant-based materials used to generate nanoparticles that not only show good biocompatibility but also have bio-degradation properties with an added advantage for targeted delivery of antigens. Such nanoparticles can protect antigens from degradation, increase their bio-availability and enable controlled release, hence improving the immune response. First the exhaustive researches were dedicated efforts, plant-based nanoparticles loaded with antigen characterization, stability studies under changing conditions, release kinetics bioactivity and immunogenicity. Properties as nanoparticle composition, method of encapsulation and the presence of stabilizers or adjuvants do play a key role in stability assessment. The application of plant-derived nanoparticles demonstrates great promise as sustainable oral vaccines for fish, due to their capacity to encapsulate a variety of bacterial, viral and parasitic antigens which can be directed against different infectious diseases that affect aquaculture species. Moreover, their multivalent and combination vaccine delivery potential as well as the versatility regarding routes of administration including mucosal/oral immunizations makes these nanoparticle-based formulations highly appealing.

Outlook and Future Research Opportunities

The progress in the plant-based nanoparticle based fish vaccines development and implementation can be defined as a significant one so far, but there are still numerous challenges. These formulations are postulated to translate well into scale-up and manufacturing, however further work needs to be done in addressing regulatory aspects of these platforms alongside safety considerations. Future directions of research should include the discovery of alternative plant sources, design and engineering targeted delivery systems incorporating modern methods for characterization along with omics based approaches. These efforts should also focus on the enhancement of stability, controlled-release and bio-availability properties by using innovative encapsulation procedures (micro or nano) as well as surface modifications allowing for their release in a specific target dictionary. This will require interdisciplinary collaborations and stakeholder partnerships between researchers, industry and regulatory authorities to address these obstacles together which are crucial for future success of plant-based nanoparticles in the delivery of effective preventive fish vaccines on a broad scale. This highlights the potential applications as well challenges faced by plant-based nanoparticles in aquaculture and provides insights for enabling research direction to harness advantages of nano-carriers towards economically viable, safe vaccination procedures optimized against major pathogens on which improvements are needed for achieving sustainability along with growth goals.

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